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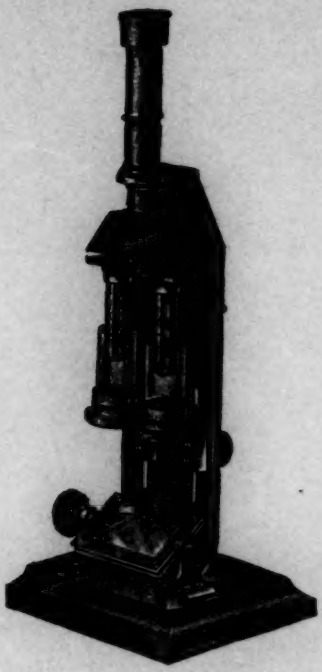

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THE DIGESTIBILITY OF CHICKEN SKIN.

By EDWARD F. KOHMAN and H. A. SHONLE.

(From the Food Research Laboratory, Bureau of Chemistry, Department of Agriculture, Indianapolis.)

(Received for publication, February 3, 1920.)

While certain feeding experiments were being conducted in poultry fattening by the use of various feed by-products, an opportunity presented itself to collect a considerable quantity of chicken skin. Advantage was taken of this to carry out two metabolism experiments to determine the digestibility of this food product. From 10 to 15 per cent of the edible nitrogen in the chicken is found in the form of skin. The fat content of this skin may vary from a very small percentage to as high as 50 per cent or even more, counting the loose layers of fat lying next to the muscular tissue as part of the skin, as was done in the experiments alluded to above.

In order to remove part of this fat to make the skin more suitable to the metabolism experiments, the ground skin was heated in a water bath and then the larger part of the fat was expressed through a cloth bag. In this manner considerable water extract was also expressed, which gelatinized under the layer of fat upon cooling. This extract was combined with the skin from which it had been expressed and the whole thoroughly mixed again. The skin as then used for the experiments had 26.3 per cent fat and 3.03 per cent nitrogen. To prepare it for eating it was rolled in balls and fried. In this form it was a fairly palatable dish. It can be calculated from Tables I and II that during the experimental period this skin furnished 67.5 per cent of the nitrogen in the diet for Subject A and 65.1 per cent for Subject B.

It should be said of the chicken skin used in the experiments that it came from broilers and roasters as they came from the

range to the feeding station and from similar birds after a 2 weeks period of fattening on a buttermilk mash. The fattened birds were in the majority, and, as they were all commercially dry picked, the skin had an abundance of pin feathers.

The details of the experiments are given in Tables I to V. Each period consisted of 5 days, the experimental period being preceded and followed by a like period with the exception that milk, eggs, and meat took the place of the chicken skin. Other-

TABLE I.

Food Intake, Subject A, Man, Weight 120 Lbs.

Food.	Fore period.		Experimental period.		After period.	
	Food.	Nitrogen.	Food.	Nitrogen.	Food.	Nitrogen.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Apples	1,050	0.26	1,050	0.26	1,050	0.26
Prunes	450	0.50	450	0.50	450	0.50
Oranges	1,189	1.53	1,189	1.53	1,213	1.57
Lettuce	240	0.46	200	0.38	200	0.38
Tomatoes	500	0.96	500	0.96	500	0.96
Sugar	514		324		108	
Tapioca	73	0.02	73	0.02	73	0.01
Bread	616	8.38	550	7.92	550	7.34
Potatoes	1,877	6.61	1,877	6.61	1,877	6.61
Butter	412	0.66	135	0.22	194	0.31
Eggs	496	10.61				
Meat	575	16.71			1,156	38.18
Milk	2,486	11.61				
Skin			1,260	38.18		
Total N		58.31		56.58		56.12
Total calories	14,697		11,589		8,792	

wise the diet was kept uniform throughout and an attempt was made to have it approximately normal for the individuals concerned. No attempt was made to limit the intake. As a result the caloric intake for the fore period was considerably higher than the after period due in part to the novelty of the experiment and the cold weather in the beginning, in part to the high fat content of the first lot of Hamburger steak purchased, and a combination of other circumstances. The feces for the different periods

TABLE II.

Food Intake, Subject B, Man, Weight 140 Lbs.

Food.	Fore period.		Experimental period.		After period.	
	Food.	Nitrogen.	Food.	Nitrogen.	Food.	Nitrogen.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Apples	1,078	0.27	1,078	0.27	1,078	0.27
Prunes	562	0.62	562	0.62	450	0.50
Oranges	1,240	1.60	1,209	1.56	1,150	1.48
Lettuce	240	0.46	200	0.38	200	0.38
Tomatoes	500	0.96	500	0.96	500	0.96
Sugar	459		270		278	
Tapioca	85	0.02	85	0.02	85	0.01
Bread	776	10.55	686	9.88	722	9.60
Potatoes	2,403	8.48	2,403	8.48	2,403	8.48
Butter	482	0.77	371	0.59	242	0.39
Eggs	484	10.36				
Meat	670	19.45			1,285	42.42
Milk	3,820	17.23				
Skin			1,400	42.42		
Total N		70.77		65.18		64.49
Total calories	17,237		14,753		11,070	

TABLE III.

Utilization of Nitrogen.

	Subject A.			Subject B.		
	Fore period.	Experimental period.	After period.	Fore period.	Experimental period.	After period.
Urine, *cc*	6,985	8,125	9,210	7,395	6,970	8,185
Feces, *gm*	524	464	573	1,145	921	661
Urinary nitrogen, *gm*	38.33	47.69	54.04	55.30	59.90	61.99
Fecal " "	7.57	8.14	8.45	13.01	11.08	8.73
Nitrogen balance, "	+12.41	+0.75	−6.37	+2.46	−5.80	−6.11
Utilization, *per cent*	87.02	85.61	84.94	83.69	83.00	86.49

TABLE IV.

Fat Ingested.

Food.	Subject A.			Subject B.		
	Fore period.	Experimental period.	After period.	Fore period.	Experimental period.	After period.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Butter	350	115	165	410	316	206
Eggs	52			51		
Meat	109		74	128		82
Skin		332			369	
Milk	99			153		
Bread	8	7	7	10	9	9
Total	618	454	246	752	694	297

TABLE V.

Utilization of Fat.

	Subject A.			Subject B.		
	Fore period.	Experimental period.	After period.	Fore period.	Experimental period.	After period.
Dry matter in feces, *per cent*	25.91	25.64	23.28	17.85	14.96	20.86
Fat in dry matter, " "	12.80	12.17	9.84	13.44	15.06	7.84
" excreted, *gm*	17.37	14.48	13.13	27.46	20.75	10.83
" utilized, *per cent*	97.2	96.8	94.7	96.3	97.0	96.4

were marked off by means of lamp black. The vegetables and fruits were not analyzed, the composition given by Atwater and Bryant being used.[1]

CONCLUSION.

In experiments in which 65.1 to 67.5 per cent of the nitrogen of the diet was supplied by chicken skin there was as good utilization of the nitrogen as when the same proportion of the nitrogen was supplied by meat, eggs, and milk.

[1] Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Off. Exp. Stations, Bull. 28*, revised, 1906.

EFFECT OF SLEEP UPON THE ALKALI RESERVE OF THE PLASMA.

By J. B. COLLIP.

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(Received for publication, February 17, 1920.)

It has been shown by Leathes (1) that the CO_2 tension of alveolar air is definitely increased as a result of sleep. He is of the opinion that the respiratory center is depressed during sleep and that the morning alkaline tide in the urine, described by him, is due to increased respiratory activity on waking. That the respiratory center is depressed during sleep would seem quite possible. It is also to be expected, however, that with depression of the respiratory center there would occur a slight increase in the C_H of the blood. As the reaction of the blood is determined by the molecular ratio $\frac{H_2CO_3}{NaHCO_3}$ (2) it would follow, if the CO_2 tension of the alveolar air is increased without a proportionate rise in the blood bicarbonate, that the C_H of the blood would be slightly increased. In order to determine whether the plasma bicarbonate is altered during sleep, blood samples were taken for analysis by the Van Slyke method from nine students who kindly volunteered their services. The samples were taken in the evening shortly before the subjects retired and then again in the early morning, the subjects first being roused from a sound slumber. The blood was aspirated from an arm vein into a glass syringe containing a trace of potassium oxalate. It was transferred at once to a paraffined glass tube and centrifuged. The plasma was then saturated in a separating funnel with CO_2 at the tension of the alveolar air of the normal subject and the determination of the CO_2 content made by the method of Van Slyke and Cullen (3). The CO_2 bound as bicarbonate by 100 cc. of plasma was calculated and the results are shown in Table I.

TABLE I.

	CO_2 bound as bicarbonate by 100 cc. of plasma.	
	Evening. 9.30 to 11 p.m.	Morning. 6 to 7.30 a.m.
	cc.	cc.
W. W. B.	61	62
W. F. C.	68	64
E. M. C.	65	61
G. V. F.	65	62
R. E. F.	61	58
D. D. H.	65	65
E. G. K.	68	63
E. D. T.	66	63
G. F. Y.	67	67

DISCUSSION.

It was found in three cases that the bicarbonate level of the venous plasma was unaltered as a result of sleep, while in the remaining six instances there was a definite increase in this factor.

These results taken in conjunction with the observations of Leathes (1) that there is a definite rise in the alveolar CO_2 during sleep would indicate that the C_H of the blood is actually increased at this time. This increase in blood C_H may be effected in one of two ways, an increase in alveolar CO_2 without a relative increase in blood bicarbonate, or an increase in alveolar CO_2 with an actual fall in blood bicarbonate. One or the other of these types of regulation of blood C_H is manifested by the sleeping individual.

SUMMARY.

The alkali reserve of the blood plasma is either unaltered or depressed during sleep. An actual increase in the C_H of the blood during sleep is indicated.

I wish in conclusion to thank the students who offered their services, and Dr. P. L. Backus who assisted in making the analyses.

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1. Leathes, J. B., *Brit. Med. J.*, 1919, ii, 165.
2. Henderson, L. J., *Ergebn. Physiol.*, 1909, viii, 254.
3. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

THE UTILIZATION OF α-METHYLGLUCOSIDE BY ASPERGILLUS NIGER.

By ARTHUR W. DOX and G. W. ROARK, Jr.

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(Received for publication, January 27, 1920.)

The striking difference in the rate of utilization of α- and β-methylglucoside by *Aspergillus niger* was pointed out some years since by Dox and Neidig.[1]

When a culture medium containing α-methylglucoside as the only source of carbon was inoculated with spores of *Aspergillus niger*, only a very meager growth of the fungus resulted. Even after several weeks the surface of the medium was only incompletely covered with mycelium in isolated colonies. On the other hand, the isomeric β-methylglucoside gave a dense mycelium which covered the entire surface. Examination of the substrate in both cases showed that in 6 days 100 per cent of the β-glucoside had disappeared, but only 1.2 per cent of the α-glucoside. Even after 20 days the latter had diminished by only 8.2 per cent. Experiments with enzyme preparations showed a similar difference in behavior toward the two isomers. Under practically the same conditions, 5.2 per cent of the α- and 85.5 per cent of the β-glucoside were hydrolyzed.

From these experiments it is apparent that α-methylglucoside can be utilized to a slight extent by this organism and that the enzyme necessary for its hydrolysis is produced in small amount under ordinary conditions. Attempts to bring about an adaptation of the fungus by transferring the mycelium to the glucoside solution after previous growth on sucrose did not result in any marked increase in availability of the substrate. The conditions under which this experiment was conducted were not such as to induce the maximum utilization of the substrate.

[1] Dox, A. W., and Neidig, R. E., *Biochem. Z.*, 1912, xlvi, 397.

This method of bringing about an apparent adaptation of an organism to a new substrate, by allowing the organism to attain a vigorous growth then replacing the original medium by the new substrate, was first employed by Pottevin.[2] In this way, Pottevin claims to have succeeded in inducing *Aspergillus niger* to secrete in demonstrable quantities the enzymes necessary for the hydrolysis of the two isomeric methylgalactosides. In his experiments, however, the substrate contained all the elements of the original culture medium. His experiments with methylgalactoside and our previous experiments with α-methylglucoside were therefore not strictly comparable. Subsequent experiments on autolysis of this organism, conducted by one of us,[3] showed that replacement of the exhausted medium by a quantity of fresh medium resulted in a much denser growth and less autolysis than replacement of the medium by a solution of pure sucrose. It seems probable that the utilization of the new substrate depends to a considerable extent upon the presence of an available source of nitrogen, and perhaps also mineral salts.

EXPERIMENTAL.

The object of this work was to determine more precisely the extent to which *Aspergillus niger* can utilize α-methylglucoside under optimum conditions. As already stated, this substance when introduced into a culture medium as the only source of carbon, enables spores of *Aspergillus niger* to germinate and develop to a thin scanty mycelium which seems to direct most of its vitality toward the formation of spores. α-Methylglucoside is, however, not toxic since its presence in a medium containing sufficient sugar has no inhibitory effect upon growth. The following experiments demonstrate this fact quite clearly.

A culture medium was prepared according to Czapek,[4] omitting the sucrose. 50 cc. of this were placed in each of three 200 cc. Jena flasks. To the first was added 2 per cent of sucrose, to the second 2 per cent of α-methylglucoside, and to the third 1 per cent of each of the preceding. The flasks were sterilized, inocu-

[2] Pottevin, H., *Ann. Inst. Pasteur*, 1903, xvii, 31.
[3] Dox, A. W., *J. Biol. Chem.*, 1913–14, xvi, 479.
[4] Czapek, F., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 47.

lated with spores of *Aspergillus niger*, and placed in an electric incubator at 35° until the resulting mycelium had become covered with spores. The mycelium was then filtered on Gooch crucibles, washed, dried, and weighed.

The filtrates gave no test for reducing sugar. It is apparent from Table I that in both Nos. 2 and 3 some of the glucoside has been utilized by the fungus. Medium 3, which contained only half as much sucrose as No. 1 and half as much glucoside as No. 2, yielded more mycelium than half the sum of those from Nos. 1 and 2.

TABLE I.

Medium No.	Weight of dry fungus.
	gm.
1	0.2779
2	0.0729
3	0.1830

The above experiment was repeated, using Raulin's medium upon which this organism thrives still better. No. 1 contained no glucoside, No. 2 contained 2 per cent of glucoside in place of the sucrose, and No. 3 contained 1 per cent of glucoside in addition to the full amount of sucrose.

TABLE II.

Medium No.	Weight of dry fungus.		
	1	2	Average.
	gm.	*gm.*	*gm.*
1	0.5906	0.5720	0.5813
2	0.0715	0.0851	0.0783
3	0.6970	0.7203	0.7087

The amount of glucoside utilized is evidently greater in No. 3 than in No. 2 (Table II). The probable explanation for this is the fact that after the fungus has attained a vigorous growth on the sucrose there is a much greater amount of mycelium to attack the glucoside than in the case of the scanty growth without the sucrose. Further experiments, recorded in Table III, apparently failed to confirm this observation, because the utilization of gluco-

side was carried practically to completion in all cases. The increase due to 1 per cent of glucoside is just about half the yield from 2 per cent of glucoside alone.

The effect of cultivating the fungus on Raulin's medium until vigorous growth and then replacing the medium by a similar medium in which α-methylglucoside is substituted, is quite striking. In the experiments recorded in Table IV the fungus

TABLE III.

Medium No.	Weight of dry fungus.					
	1	2	3	4	5	Average.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.9115	0.8924	0.8112	0.8574	0.8552	0.8655
2	0.1606	0.1423	0.1474	0.1421	0.1417	0.1468
3	1.0206	0.9755	0.9014	0.9045	0.8955	0.9393

TABLE IV.

Age of culture after transfer.	Before spore formation.		After spore formation.	
	Rotation of medium.	Decrease.	Rotation of medium.	Decrease.
days	°V.*	*per cent*	°V.	*per cent*
0	17.5	0.0	18.2	0.0
1	14.2	18.8	13.6	25.1
2	13.6	22.2	12.2	32.9
3	12.2	30.2	11.4	37.5
4	11.8	32.5	10.8	40.6
5	11.6	33.7	10.0	45.0
6	11.2	36.0	8.8	51.6
7	11.0	37.1	6.8	61.5
8	11.0	37.1	7.0	62.6

*°V. = degrees on Ventzke scale. For conversion to angular degrees multiply by 0.3468.

was first grown on Raulin's medium. In the first series the transfer was made just before and in the second series just after the formation of spores. The transfer of media was made by first syphoning off the medium and replacing twice with equal amounts of sterile distilled water, each time allowing the mycelium and water to remain in contact about 45 minutes, taking care not to wet the surface of the culture, and finally replacing the water

with medium containing glucoside as the sole source of carbon. On successive days the medium was removed from one of these flasks and an aliquot examined polarimetrically after clarification with neutral lead acetate. From the decrease in optical rotation the per cent loss of glucoside is calculated. The glucose was used up by the fungus as fast as it was liberated from the glucoside, as is shown by the fact that the test with Fehling's solution was invariably negative. Qualitative tests for methyl alcohol were, however, positive.

As may be seen from Table IV, the more mature cultures utilize a larger percentage of glucoside in a given time. This

TABLE V.

Age of culture after transfer.	Before spore formation.		After spore formation.	
	Rotation of medium.	Decrease.	Rotation of medium.	Decrease.
days	°V.	*per cent*	°V.	*per cent*
0	17.9	00.0	17.7	00.0
1	13.4	25.1	14.0	20.9
2	11.2	37.4	12.4	29.9
3	9.0	49.7	11.6	34.4
4	9.0	49.7	10.6	40.1
5	7.0	60.9	7.8	56.4
6	5.8	67.6	5.2	70.6
7	4.4	75.4	4.8	72.1
8	2.8	84.3	3.8	78.4
9	2.0	88.9	1.6	90.9
10	1.8	89.9	1.4	92.1

is in accord with our previous results, since the older cultures in this case contained more actual vegetative mycelium. The difference in utilization is, however, greater than can be assigned to this cause alone.

A similar experiment was conducted to see if the presence of α-methylglucoside in the original medium would lead to a greater response. It was carried out under as nearly the same conditions as possible.

Table V shows that there is not so great a difference in the ability to utilize the glucoside before and after spore formation when the culture is first grown on a medium containing glucoside. This seems to indicate an adaptation of *Aspergillus niger* to

α-methylglucoside. Control flasks containing the sterile medium were carried along with these experiments. In every case there was a slight increase in rotation likely due to evaporation. This showed that there was no hydrolysis or molecular rearrangement of the glucoside due to standing at that temperature for a number of days.

Another attempt at adaptation of *Aspergillus niger* to α-methylglucoside was conducted in the following manner. A culture medium containing both sucrose and α-methylglucoside was prepared and distributed in a number of test-tubes, all of which were then sterilized. Inoculations were made successively from one tube to another as soon as the culture reached maturity. After cultivating the mold for nine generations on this medium the tenth transfer was made to 50 cc. of a medium containing

TABLE VI.

Age of culture.	Control.		Attempted adaptation.	
	Rotation of medium.	Decrease.	Rotation of medium.	Decrease.
days	°*V.*	*per cent*	°*V.*	*per cent*
0	17.8	0.0	17.8	0.0
6	17.0	4.4	16.8	5.6
6	17.0	4.4	17.0	4.4

glucoside but no sucrose. As a control, a parallel culture which had been propagated for the same number of generations on sucrose alone was transferred to another 50 cc. of the glucoside medium. The results are given in Table VI.

The extent of adaptation, if any, is so slight after nine successive generations of *Aspergillus niger* grown in the presence of glucoside as to be within the range of normal cultural variations.

SUMMARY.

Aspergillus niger grows very poorly on media containing α-methylglucoside as the only source of carbon, but readily on sucrose media in the presence of the glucoside. A vigorous culture transferred entire to the glucoside medium without sucrose may use up the glucoside more rapidly than a culture obtained by

direct inoculation of this medium with spores. If the original medium contained both sucrose and glucoside the latter disappears more rapidly from the second medium containing glucoside but no sucrose than when the original medium contained sucrose alone. Also there was a slight difference between the activity of cultures before and after spore formation.

A gradual cumulative adaptation through a number of generations cultivated on this substrate could not be demonstrated with any degree of certainty.

PROPERTIES OF THE NUCLEOTIDES OBTAINED FROM YEAST NUCLEIC ACID.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 4 AND 5.

(Received for publication, March 1, 1920.)

The four nucleotides composing the molecule of yeast nucleic acid have now been isolated in crystalline form. Of these adenosinphosphoric acid was isolated by different methods by Jones and Kennedy[1] and by Levene.[2] The two substances seemed to differ from one another on the point of the crystal water.

Cytidinphosphoric acid also was obtained in two laboratories (Thannhauser and Dorfmüller[3] and Levene[4]) and the two substances seemed to differ in their optical activity. Also the two samples isolated by the present writer showed minor differences in their rotatory power. The properties of guanosinphosphoric[5] and uridinphosphoric acids[6] had been given as found on substances as they were first obtained without further recrystallization.

The present work was undertaken for the sake of clearing up the above mentioned discrepancies and, further, for the sake of establishing the composition of the nucleotide as regards the crystal water, and for the sake of establishing the physical constants with greater rigor. In order to make the work possible, larger quantities of the crude material were required and were prepared. The results of the analysis of the carefully purified substance are as follows:

[1] Jones, W., and Kennedy, R. P., *J. Pharm. and Exp. Therap.*, 1919, xiii, 45.

[2] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 415.

[3] Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1919, 104, 5.

[4] Levene, P. A., *J. Biol. Chem.*, 1920, xli, 19.

[5] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

[6] Levene, P. A., *J. Biol. Chem.*, 1920, xli, 1.

Adenosinphosphoric acid (Fig. 1), according to Jones and Kennedy,[1] crystallizes with 1 molecule of crystal water. Levene, in his original work, found the air-dry substance anhydrous. The observation could not be repeated. Many samples have been analyzed since the first publication and all contained 1 molecule of crystal water. This was easily removed by drying under diminished pressure at the temperature of xylene vapor for 24 hours. The original sample was reanalyzed and again found practically anhydrous (1 per cent of water). In a sealed capillary tube the air-dry substance decomposed with effervescence at 195°C. (corrected).

Guanosinphosphoric acid (Fig. 2) crystallized in long needles of the appearance of the crystals of guanosin. It crystallized with 2 molecules of crystal water, which could be removed completely on drying under diminished pressure at the temperature of xylene vapor. In a sealed capillary tube the air-dry substance softened and became semitransparent at 175°C. and melted at 180°C. (corrected).

Uridinphosphoric acid (Fig. 3) was recrystallized out of methyl alcohol. The crystals formed slowly on evaporation of the alcohol. The larger crystals were often superimposed by deposits of smaller crystals, hence the photographic reproduction of the crystals was difficult. Besides, the yield of the material was rather small. For this reason it was decided to measure the optical rotation also of the monoammonium salt, which has good physical properties and a convenient solubility. The free acid crystallized in elongated prisms with pointed ends and melted in a sealed capillary tube at 198.5°C.

The monoammonium salt crystallized in prismatic needles which contained no crystal water. The air-dry substance heated in a sealed tube contracted and turned semitransparent at 200°C. (corrected), and decomposed at 242°C.

Cytidinphosphoric acid (Fig. 4) on repeated recrystallizations appeared in form of elongated plates which contained no crystal water. In a sealed capillary tube the air-dry substance decomposed with effervescence at 230–233°C.

Rotations.

The rotations were measured: (*a*) in aqueous solution, (*b*) in a solution of 10 per cent hydrochloric acid, (*c*) in a 5 per cent aqueous solution of ammonia, (*d*) in a 2 per cent aqueous solution of caustic soda, and (*e*) in a 5 per cent solution of the same.

Guanosin- and adenosinphosphoric acids showed a minimum levorotation in hydrochloric acid solution, which increased in water and in aqueous ammonia, and reached a maximum in an aqueous solution of sodium hydroxide.

Cytidinphosphoric acid showed a maximum dextrorotation in aqueous solution, successively decreasing in aqueous ammonia, hydrochloric acid, 2 per cent sodium hydroxide, and turned levorotary in 10 per cent sodium hydroxide.

Uridinphosphoric acid and its ammonium salt changed their optical rotation in the same direction as the preceding substance.

The changes in the optical rotation probably are the resultants of more than one factor. Among these the tautomeric changes in the basic radical of the substance possibly play an important part. Indeed, adenosin and uridin showed the same character of optical rotation as the corresponding nucleotides. On the other hand, the molecular rotation of the nucleoside and of the nucleotide are not identical.

EXPERIMENTAL.

Guanosinphosphoric Acid.—10 gm. of the crystalline material (No. 26) described in a previous communication[5] were recrystallized out of water. One part of the substance was suspended in thirty parts of water and the water kept boiling until solution was completed. The solution was allowed to stand at room temperature (about 25°C.) over night. The yield of recrystallized material was 7.5 gm. (No. 291). It was planned to continue recrystallization until a constant optical rotation of the substance was attained. On the first recrystallization the specific rotation remained without change. No. 291 was recrystallized once more out of 200 cc. of water in the same manner as No. 26. The resulting material showed no change in its optical rotation. The air-dry substance heated in a sealed capillary tube contracted and turned semitransparent at 175°C. (corrected) and decomposed at 180°C.

0.1168 gm. of the air-dry substance on drying to constant weight under diminished pressure at the temperature of xylene vapor lost 0.1112 gm. in weight.

	Calculated for $C_{10}H_{14}N_5PO_8 + 2H_2O$.	Found.
	per cent	*per cent*
H_2O	9.47	9.67

The dry substance (No. 312) analyzed as follows:

0.1056 gm. of the substance gave 0.1312 gm. of CO_2 and 0.0168 gm. of H_2O.

0.1807 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 34.85 cc. of 0.1 N acid.

0.2710 gm. of the substance gave 0.0839 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_8$.	Found.
	per cent	*per cent*
C	33.05	33.89
H	3.89	3.90
N	19.22	19.25
P	8.55	8.62

Rotation of the substance in aqueous solution:

$$\text{No. 26} \qquad [\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5°$$

$$\text{No. 291} \qquad [\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5°$$

$$\text{No. 312} \qquad [\alpha]_D^{25} = \frac{-0.16 \times 100}{1 \times 2} = -8.0°$$

Rotation of the air-dry substance in 10 per cent hydrochloric acid:

$$\text{No. 312} \qquad [\alpha]_D^{25} = \frac{+0.03 \times 100}{1 \times 2} = +1.5°$$

Rotation of the air-dry substance in 5 per cent aqueous ammonia solution:

$$\text{No. 312} \qquad [\alpha]_D^{20} = \frac{-0.88 \times 100}{1 \times 2} = -44.0°$$

The rotation of the substance in 2 per cent aqueous caustic soda was as follows:

$$[\alpha]_D^{25} = \frac{-1.14 \times 100}{1 \times 2} = -57.0°$$

In 5 per cent of the same:

$$[\alpha]_D^{25} = \frac{-1.30 \times 100}{1 \times 2} = -65.0°$$

Adenosinphosphoric acid is the most insoluble of the four nucleotides. 23 gm. of the substance (No. 286) were suspended in 1 liter of hot water and the water was kept boiling for some time and since the substance did not dissolve readily dilute ammonia was added gradually until solution was completed. The hot solution was rendered slightly acid to litmus by means of acetic acid and allowed to stand at room temperature over night. A crystalline deposit (No. 282) formed.

The precipitate thus formed was recrystallized out of 600 cc. of water and allowed to crystallize over night. A crystalline deposit was formed. The yield was 7.5 gm. (No. 290). These were again recrystallized out of hot water. The yield was 5 gm. (No. 306). On heating in a sealed capillary tube the substance decomposed at 195°C. (corrected).

Analysis of the substance:

0.1325 gm. of the substance on drying under diminished pressure at the temperature of xylene vapor lost 0.0069 gm. of water.

	Calculated for $C_{10}H_{14}N_5PO_7 + H_2O$.	Found.
	per cent	*per cent*
H_2O	4.90	5.20

Several samples were analyzed with the same result whereas the first two samples were analyzed for the anhydrous substance.

The dry substance (No. 306) analyzed as follows:

0.1256 gm. of the substance gave on combustion 0.1599 gm. of CO_2 and 0.0456 gm. of H_2O.

0.0948 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 13.56 cc. of 0.1 N acid.

0.2844 gm. of the substance gave 0.0913 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7$.	Found.
	per cent	*per cent*
C	34.57	34.71
H	4.07	4.06
N	20.17	20.13
P	8.94	9.04

Rotation of the substance in 1 per cent aqueous solution in 2 dm. tube:

$$\text{No. 306} \qquad [\alpha]_D^{20} = \frac{-0.81 \times 100}{2 \times 1} = -40.5°$$

Since the substance is only little soluble in water the readings of the substances in process of their purification were taken in 5 per cent aqueous ammonia.

Rotation in 5 per cent aqueous ammonia solution:

$$\text{No. 282} \qquad [\alpha]_D^{20} = \frac{-0.81 \times 100}{1 \times 2} = -40.5°$$

$$\text{No. 290} \qquad [\alpha]_D^{20} = \frac{-0.89 \times 100}{1 \times 2} = -44.5°$$

$$\text{No. 306} \qquad [\alpha]_D^{20} = \frac{-0.83 \times 100}{1 \times 2} = -41.5°$$

The slight variation might be due to variation in the moisture of the substance.

Rotation in a solution of 10 per cent hydrochloric acid:

$$\text{No. 306} \qquad [\alpha]_D^{20} = \frac{-0.76 \times 100}{1 \times 2} = -38.00°$$

The rotation of the substance in 2 per cent aqueous caustic soda was as follows:

$$[\alpha]_D^{20} = \frac{-1.19 \times 100}{1 \times 2} = -59.5°$$

In 5 per cent of the same:

$$[\alpha]_D^{20} = \frac{-1.32 \times 100}{1 \times 2} = -66.0°$$

Cytidinphosphoric Acid.—The starting material was a mixture of cytidin- and adenosinphosphoric acid, prepared in the manner described in the previous paper. The original optical rotation was $[\alpha]_D^{20} = +25$. By recrystallization out of 50 per cent alcohol finally a substance was obtained with $[\alpha]_D^{20} = +40$. 10.0 gm. of this material (No. 270) were dissolved in 500 cc. of boiling water and to the solution 500.0 cc. of 99.8 per cent alcohol were added. Soon heavy crystals began to settle out and the crystallization was allowed to proceed 48 hours. The yield of the crystals was 7.5 gm. (No. 280). This material was then dissolved in

400 cc. of boiling water and 400 cc. of 99.8 per cent alcohol were added to the solution. The crystallization proceeded as above. The yield of the crystals was 6.0 gm. (No. 285). These 6.0 gm. were dissolved in 350 cc. of boiling water and to the solution 150.0 cc. of 99.8 per cent alcohol were added. The yield of the final material (No. 289) was 5.0 gm. The crystal form is reproduced in Fig. 4.

The substance in sealed capillary tube decomposed with effervescence at 230–233°C. (corrected). (Heating slow.)

The analysis of the substance was as follows:

On drying under diminished pressure at the temperature of xylene vapor the substance lost 0.8 per cent in weight.

0.1123 gm. of the dry substance gave 0.1374 gm. of CO_2 and 0.0442 gm. of H_2O.

0.0992 gm. of the dry substance employed for Kjeldahl nitrogen estimation required for neutralization 9.23 cc. of 0.1 N acid.

0.2976 gm. of the substance gave 0.1014 gm. of $Mg_2P_2O_7$.

	Calculated for $C_6H_{14}N_2PO_8$.	Found.
	per cent	*per cent*
C	33.42	33.36
H	4.37	4.41
N	13.00	13.03
P	9.61	9.50

The optical rotation was as follows:

In aqueous solution:

$$\text{No 270} \quad [\alpha]_D^{20} = \frac{+\ 0.80 \times 100}{1 \times 2} = +\ 40.0°$$

$$\text{No. 285} \quad [\alpha]_D^{20} = \frac{+\ 0.97 \times 100}{1 \times 2} = +\ 48.5°$$

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+\ 0.95 \times 100}{1 \times 2} = +\ 47.5°$$

In 10 per cent hydrochloric acid solution:

$$\text{No. 289} \quad [\alpha]_D^{25} = \frac{+\ 0.52 \times 100}{1 \times 2} = +\ 26.0°$$

In 5 per cent ammoniacal solution:

$$\text{No. 289} \quad [\alpha]_D^{25} = \frac{+\ 0.89 \times 100}{1 \times 2} = +\ 44.5°$$

In 2 per cent sodium hydroxide solution:

$$\text{No. 289} \qquad [\alpha]_D^{20} = \frac{+ 0.51 \times 100}{1 \times 2} = + 25.5°$$

In 5 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{+ 0.02 \times 100}{1 \times 2} = + 1.0°$$

In 10 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.42 \times 100}{1 \times 2} = - 21.0°$$

Uridinphosphoric Acid.—The material described in a previous communication was dissolved in boiling methyl alcohol and the solution was allowed to stand in a desiccator over sulfuric acid. On standing, after a considerable part of the alcohol evaporated, heavy crystals settled out on the walls of the dish. The crystallization proceeded very slowly.

The substance melted in sealed tube with decomposition at M.P. = 198.5°C. (corrected).

The analysis of the substance was as follows:

0.1000 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 5.97 cc. of 0.1 N acid.

	Calculated for $C_9H_{13}N_2PO_9$.	Found
	per cent	*per cent*
N	8.64	8.36

The rotation of the substance (No. 343) was as follows:

In aqueous solution:

$$[\alpha]_D^{20} = \frac{+ 0.19 \times 100}{1 \times 2} = + 9.5°$$

In 2 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{+ 0.13 \times 100}{1 \times 2} = + 6.5°$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 0.30 \times 100}{1 \times 2} = - 15.0°$$

Monoammonium Salt of Uridinphosphoric Acid.—The crystalline material described in a previous communication was dissolved in a minimum amount of water and to the solution 20 volumes of methyl alcohol were added. The solution was allowed to stand at room temperature. After several days on walls of the flask a sediment formed, which consisted of fine curved felt-forming needles (No. 254). In the mother liquor, on further standing, a third precipitate formed. The crystal form is reproduced in Fig. 3.

The substance when heated in a sealed capillary tube contracted and turned semitransparent at 200°C. (corrected) and decomposed with effervescence at 240°C.

The analysis of the substance was as follows:

0.1204 gm. of the substance gave on combustion 0.1417 gm. of CO_2 and 0.0514 gm. of H_2O.

0.1964 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 17.24 cc. of 0.1 N acid.

0.1964 gm. of the substance gave 0.0650 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{13}N_2PO_8$.	Found.
	per cent	*per cent*
C	31.66	32.09
H	4.73	4.77
N	12.32	12.30
P	9.58	9.15

The optical rotation of the substance was as follows:

In aqueous solution:

$$\text{No. 255} \qquad [\alpha]_D^{30} = \frac{+\ 0.21 \times 100}{1 \times 2} = +\ 10.5°$$

In 10 per cent hydrochloric acid solution:

$$\text{No. 255} \qquad [\alpha]_D^{25} = \frac{+\ 0.05 \times 100}{1 \times 2} = +\ 2.5°$$

In 5 per cent ammoniacal solution:

$$\text{No. 255} \qquad [\alpha]_D^{25} = \frac{+\ 0.28 \times 100}{1 \times 2} = +\ 14.0°$$

The rotation of the substance in 2 per cent aqueous sodium hydroxide was:

$$[\alpha]_D^{20} = \frac{+ 0.03 \times 100}{1 \times 2} = + 1.5°$$

In 5 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.32 \times 100}{1 \times 2} = - 16.0°$$

In 10 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.52 \times 100}{1 \times 2} = - 26.0°$$

The optical rotation of adenosin was as follows:
In aqueous solution:

$$[\alpha]_D^{20} = \frac{- 1.20 \times 100}{1 \times 2} = - 60.0°$$

In 10 per cent solution of hydrochloric acid:

$$[\alpha]_D^{20} = \frac{- 0.87 \times 100}{1 \times 2} = - 43.5°$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 1.37 \times 100}{1 \times 2} = - 68.5°$$

The optical rotation of uridin was as follows:
In aqueous solution:

$$[\alpha]_D^{20} = \frac{+ 0.08 \times 100}{1 \times 2} = + 4.0°$$

In 10 per cent solution of hydrochloric acid:

$$[\alpha]_D^{20} = \frac{+ 0.10 \times 100}{1 \times 2} = + 5.0°$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 0.12 \times 100}{1 \times 2} = - 6.0°$$

EXPLANATION OF PLATES.

PLATE 4.

FIG. 1. Crystals of adenosinphosphoric acid.
FIG. 2. Crystals of guanosinphosphoric acid.

PLATE 5.

FIG. 3. Crystals of monoammonium salt of uridinphosphoric acid.
FIG. 4. Crystals of cytidinphosphoric acid.

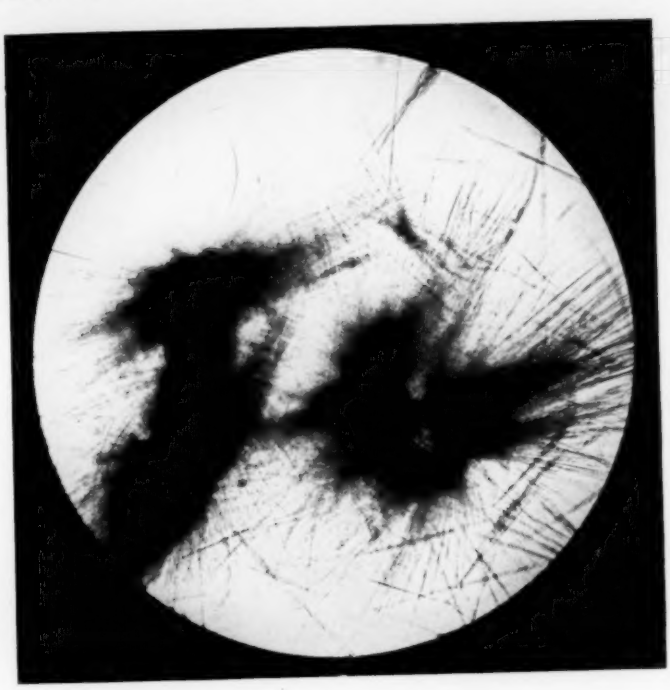

FIG. 1.

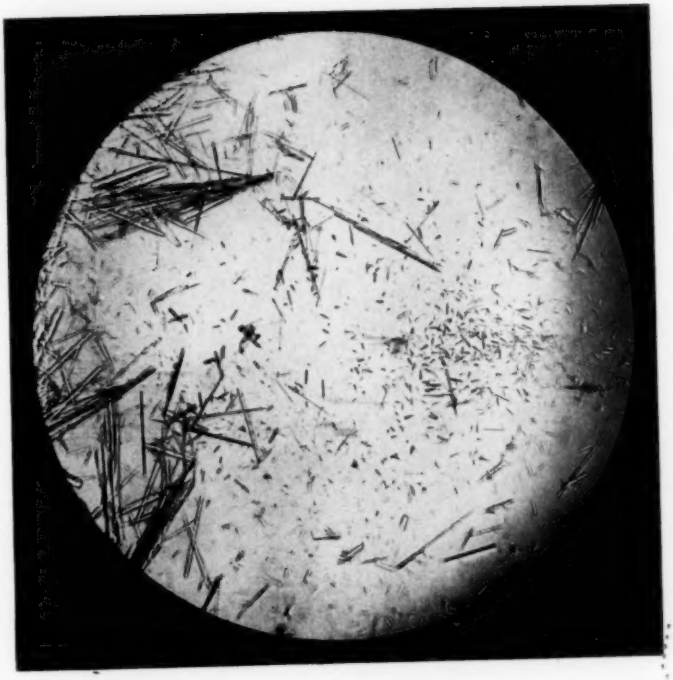

FIG. 2.

(Levene: Nucleotides from yeast nucleic acid.)

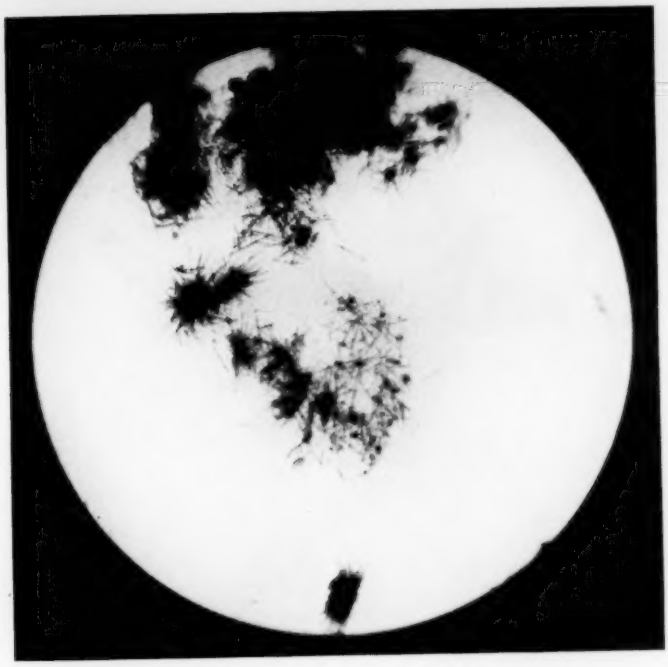

FIG. 3.

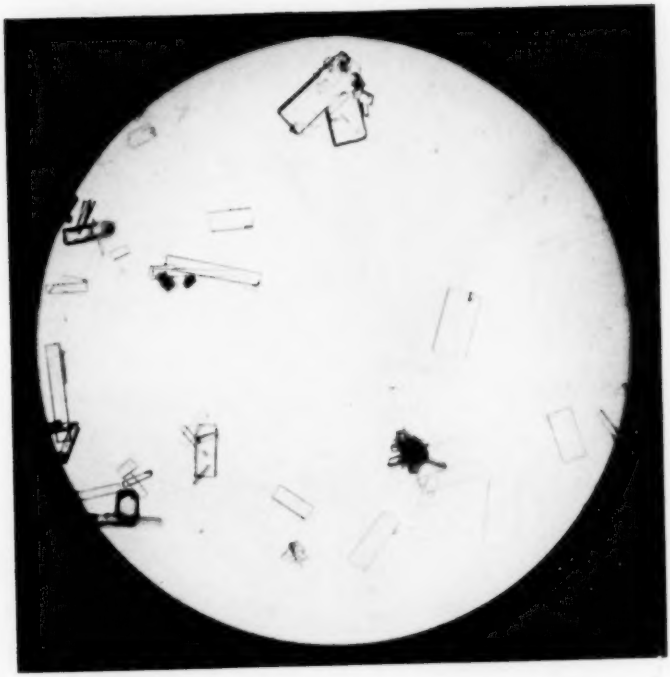

FIG. 4.

(Levene: Nucleotides from yeast nucleic acid.)

RUTIN, THE FLAVONE PIGMENT OF ESCHOLTZIA CALIFORNICA CHAM.*

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Plates 6 and 7.

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The simple chemical relationship between the flavonol and anthocyanin series of plant pigments, suggested by Combes[1] and Everest,[2] but not proved until Willstätter and Mallison[3] actually produced cyanidin from quercetin by reduction in acid solution, has led to considerable speculation as to the genetical and physiological interrelations of these compounds. There is some evidence that the anthocyanins are produced in the plant from the corresponding flavonols, and not by direct synthesis. Everest,[4] for example, has shown that glucosides of the chemically related pair myricetin and delphinidin occur side by side in purple-black forms of *Viola*. Before far reaching conclusions are drawn, however, it will be necessary to isolate, or otherwise identify, the pigments of a large number of species. It goes without saying that the best material for this purpose will be afforded by species whose color varieties are capable of genetic analysis or by species in which the relations between flavone and anthocyanin are capable of experimental modification.

The genus *Escholtzia*, abundantly distributed in California, and common in cultivation, contains garden forms with yellow, golden yellow, pale yellow, white, carmine, and rose flowers. It

*Published by permission of the Secretary of Agriculture.

[1] Combes, R., *Compt. rend. Acad.*, 1913, clvii, 1002, 1454.

[2] Everest, A. E., *Proc. Roy. Soc. London, Series B*, 1913–14, lxxxvii, 444.

[3] Willstätter, R., and Mallison, H., *Sitzungsb. kais. Akad. Wissensch.*, 1914, 769.

[4] Everest, A. E., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 251.

would provide ideal material for combined genetical and biochemical investigation providing the pigments concerned were present in large quantity and easily isolable. With this idea in mind, the writers obtained for preliminary work a large quantity of petals of wild *Escholtzia*, gathered by Mr. W. W. Wagener in the vicinity of Palo Alto, California. We have called the material *Escholtzia californica* Cham., using this specific name in the broad sense, for it was of course out of the question to observe close specific or varietal differences in gathering wild material of this polymorphic genus.

Preparation and Properties of Rutin.

The air-dried petals afforded an abundant yield of the glucoside rutin, quercetin glucoso-rhamnoside. They were first extracted for several days with ether, to remove fats, carotinoids, *etc.*, and then with ethyl alcohol. The alcoholic solution was evaporated to small bulk, poured into water, and the remaining alcohol boiled off. The crude rutin, which came down as a copious crystalline precipitate, was collected on a Buchner funnel, washed with water, dried, extracted with ether until no colored impurities were removed, and finally purified by recrystallization from a large volume of hot water. By this method 7.13 gm. of rutin (dried at 140°C.) were obtained from 150 gm. of air-dried petals.

According to Perkin and Everest,[5] rutin "is said to melt above 190°." Schmidt[6] gives 188–190°. Our preparation began to sinter at 186° and melted at 190–192°C. (uncorrected). The color of the anhydrous compound (dried at 150–160°), as ascertained by comparison with Ridgway's standards,[7] was primrose-yellow; the streak was pale green-yellow. As obtained by crystallization from hot water *Escholtzia* rutin formed microscopically fine, very dense, acute-based tufts of silky crystals (Fig. 1).

Water of crystallization was determined by exposing a sample of the glucoside to a moist atmosphere under a bell jar until it

[5] Perkin, A. G., and Everest, A. E., The natural organic colouring matters, London and New York, 1918, 197.

[6] Schmidt, E., *Arch. Pharm.*, 1908, ccxlvi, 214.

[7] Ridgway, R., Color standards and color nomenclature, Washington, 1912.

came to constant weight. It was then dried at 160° for 12 hours. The loss of water from 2.2548 gm. of glucoside was 0.1868 gm., or 8.28 per cent, according satisfactorily with the 8.13 per cent calculated from $C_{27}H_{30}O_{16}.3H_2O$, the accepted formula of rutin.

Identification of Quercetin.

Hydrolysis resolves rutin into one molecule each of quercetin, rhamnose, and glucose. Our anhydrous preparation, boiled with approximately 5 per cent sulfuric acid, gave quercetin yields of 0.5354 gm. and 0.6177 gm. from samples weighing 1.0812 gm. and 1.2460 gm., respectively. These figures correspond to 49.51 and 49.57 per cent. Theory requires 49.51 per cent. The

TABLE I.

	Weight of sample.	CO_2	H_2O	C	H	O
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Rutin	0.1645	0.3204	0.0766	53.12	5.22	41.66
	0.1810	0.3528	0.0840	53.15	5.20	41.65
Quercetin	0.1694	0.3709	0.0490	59.71	3.24	37.05
	0.1759	0.3854	0.0532	59.74	3.39	36.87
Penta-acetylquercetin	0.1220	0.2634	0.0423	58.88	3.89	37.23
	0.1106	0.2388	0.0386	58.88	3.91	37.21

crystalline quercetin (Fig. 2) was washed with cold water and dried at 140°C. It conformed in physical characteristics with quercetin from other sources. The crystals were citron-yellow; the streak light greenish yellow. In order to prevent decomposition of the material below the melting point, the bath (melted acid potassium sulfate) was heated to 300° before the sample for determination of melting point was introduced. Rosenthaler[8] states that quercetin melts with partial decomposition at 310°. Our material darkened, but did not melt, between 300 and 305°. The melting point was not so sharp as might have been wished, but melting was complete at 310°. Wunderlich[9] gives 305–310°.

[8] Rosenthaler, L., Der Nachweis organischer Verbindungen, Stuttgart, 813.

[9] Wunderlich, A., *Arch. Pharm.*, 1908, ccxlvi, 224, 241, 256.

A satisfactory identification of the compound was secured, however, by acetylation. It gave penta-acetylquercetin, melting at 189–191°. Perkin and Hummel[10] found 190–191°. Samples of the latter, weighing 0.7638 and 0.5704 gm., hydrolyzed by hydrochloric acid in glacial acetic acid, gave quercetin yields of 0.4512 and 0.3376 gm., corresponding to 59.07 and 59.18 per cent. Theory requires 58.98 per cent. Combustions were made of rutin and of the quercetin and acetylquercetin derived from it. The results, concordant and agreeing well with expectation, are given in Tables I and II.

When treated with sulfuric acid in boiling glacial acetic acid, quercetin forms a finely crystalline orange-vermilion acid addition product, $C_{15}H_{10}O_7.H_2SO_4$, from which the quercetin is easily regenerated by simple suspension in water. A sample of the

TABLE II.

	Rutin.		Quercetin.		Penta-acetylquercetin.	
	Found.	Expected.	Found.	Expected.	Found.	Expected.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C.....	53.14	53.10	59.73	59.59	58.88	58.59
H.....	5.21	4.95	3.32	3.34	3.90	3.90
O.....	41.65	41.95	36.95	37.07	37.22	37.51

sulfate weighing 0.2714 gm., dried at 100°, gave 0.2034 gm. of recovered quercetin, or 74.94 per cent. Theory requires 75.5 per cent.

The Sugars.

In view of the perfect agreement of the analytical results with the figures for rutin, complete identification required only the determination of the sugars resulting from hydrolysis. The literature of rutin and its synonyms, violaquercetrin, osyritrin, myrticolorin, *etc.*, shows that for many years the occurrence of glucose in the presence of rhamnose was overlooked, or *vice versa*. Following a modification of Perkin's procedure,[11] we separated pure glucosazone and rhamnosazone from the mixed osazones,

[10] Perkin, A. G., and Hummel, J. J., *J. Chem. Soc.*, 1896, lxix, 1295.
[11] Perkin, A. G., *J. Chem. Soc.*, 1910, xcvii, 1776.

using the differential solubility of the compounds in acetone for the primary separation, following with recrystallization from 5 per cent pyridine in water and then from 20 per cent alcohol for final purification. In this manner rhamnosazone, melting at 181–182°, and glucosazone, melting at 205–207°, were obtained. Recrystallized from 20 per cent alcohol, under identical conditions, the crystal forms were characteristically different, as shown in Figs. 3 and 4. We were unable to distinguish two types of crystals in the mixture of osazones before fractionation by acetone, but after purification the glucosazone formed typical radiate groups of short, unbranched needles, and the rhamnosazone bifasciculate clusters of longer, more slender, branching needles.

Distribution of Rutin.

All the plants which have been reported to contain rutin have been critically examined in recent years either by Schmidt[6] and Wunderlich[9] or Perkin.[11] Adding *Escholtzia* to the list, the known distribution of rutin is now as follows:

Santalaceae; leaves of *Osyris compressa*,

Polygonaceae; entire herb, but chiefly the flowers, of *Fagopyrum esculentum*,

Papaveraceae; petals of *Escholtzia californica*,

Capparidaceae; flower buds of *Capparis spinosa*,

Leguminosae; flower buds of *Sophora japonica*,

Rutaceae; leaves of *Ruta graveolens*,

Violaceae; flowers of *Viola tricolor*,

Myrtaceae; leaves of *Eucalyptus macrorhyncha*,

Globulariaceae; leaves of *Globularia alypum*.

Only *Capparis* is doubtful, the rutin from this source differing from that of *Ruta*, *etc.* in sintering 10° below the usual temperature, regardless of every effort to purify the material completely. With the exception of *Globularia*, all the plants known to contain rutin fall within the subclass Archichlamideae of the Dicotyledones.

SUMMARY.

The petals of *Escholtzia californica* contain nearly 5 per cent of rutin (quercetin glucoso-rhamnoside). In view of the great range of flower colors in *Escholtzia*, from golden yellow to white, and from white to rose, this genus would appear to afford especially suitable material for study of the physiological and genetic relationships of the flavonol and anthocyanin pigments. It is hoped that the problems will interest workers who are advantageously located for carrying out both garden and laboratory studies.

Notwithstanding the brilliant work of Willstätter in showing the chemical relation of the anthocyanins and the flavonol pigments, it is quite true, as Wheldale[12] has said, that in order to prove their relation in nature it is necessary to know which flavone accompanies which anthocyanin in a considerable number of plants. It would conserve effort in solving the problem if the flavones were isolated and identified in all the plants in which Willstätter determined the anthocyanins, and, conversely, if those plants in which the yellow pigments are well known were studied with respect to the anthocyanins. That it will be difficult to work out the relation, and that it cannot be done except by collaboration between chemists and geneticists, is shown by the fact that Sutton's "Black Knight" pansy, a variety of *Viola tricolor*, a species well known for its great range of flower colors, has been shown by Everest to contain glucosides of the pair myricetin-delphinidin, whereas one would have expected from the well established occurrence of rutin in this species that the pair quercetin-cyanidin would have been the first to be detected. The share of the geneticist in the final elucidation of the pigment situation must be to provide the chemist with material of known factorial composition.

[12] Wheldale, M., The anthocyanin pigments of plants, Cambridge, 1916, 15.

EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. *Escholtzia* rutin, crystallized from hot water (× 90).

FIG. 2. Quercetin from *Escholtzia* rutin, as obtained by hydrolysis of the rutin with boiling 5 per cent sulfuric acid (× 90).

PLATE 7.

FIG. 3. Phenylglucosazone, separated from phenylrhamnosazone by acetone and purified by recrystallization. The crystals were obtained by cooling of a hot solution in 20 per cent alcohol. Under exactly the same conditions phenylrhamnosazone crystallized as in Fig. 4 (× 90).

FIG. 4. Phenylrhamnosazone, crystallized from hot 20 per cent alcohol (× 90).

FIG. 1.

FIG. 2.

(Sando and Bartlett: Rutin.)

FIG. 3.

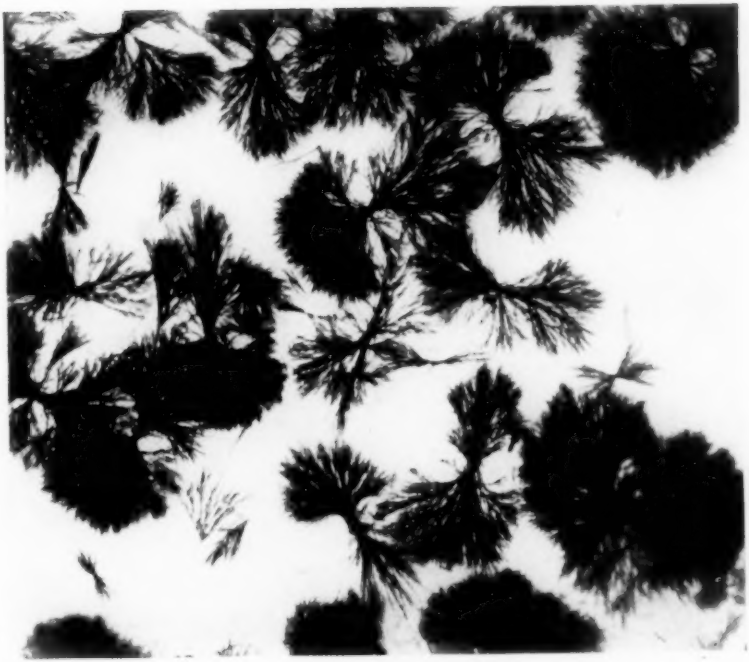

FIG. 4.

(Sando and Bartlett: Rutin.)

THE FORMATION OF ACETONE BODIES FOLLOWING ETHER ANESTHESIA AND THEIR RELATION TO THE PLASMA BICARBONATE.

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(Received for publication, February 10, 1920.)

It is a common observation that urine voided following ether anesthesia gives positive sodium nitroprusside and ferric chloride tests for acetone bodies; also, that as a result of the anesthetic there is a decrease in the CO_2-combining power of the blood plasma. Because of this coincidence it has been believed by many that the formation of acetone bodies accounts for the decreased alkaline reserve. Some observations were recently made by Reimann and Bloom (1) which appeared to confirm this theory. They studied the CO_2-combining power, together with the total blood acetone bodies, before and after ether anesthesia in a series of 60 patients and concluded that "blood acetone bodies account for 20 to 100 per cent of the bicarbonate fall observed, on an average for 60 per cent."

In the course of some work done to determine the frequency of acidosis in babies in a number of conditions, it was thought desirable to carry out some determinations of the blood acetone bodies, and for this purpose the method of Van Slyke and Fitz (2) was selected. The method is a gravimetric one and depends on the formation of a mercury-acetone complex which results from boiling mercuric sulfate, sulfuric acid, and potassium dichromate reagents in certain definite concentration with protein-free blood filtrate. Since certain substances beside acetone, including alcohol, give precipitates (2), it was necessary, if postoperative bloods were to be analyzed, to learn whether or not ether would also give a precipitate under the conditions of the determination.

This proved to be the case, the precipitate forming only after boiling with the 5 per cent potassium dichromate reagent, which was reduced, giving a green color. This precipitate was very similar in appearance to the acetone-mercury compound, and like the latter gave a positive iodoform test (Lieben's). From these preliminary observations it seemed necessary to make a study of the acetone bodies before and after anesthesia on adult cases. The general plan was to observe the relation existing between the CO_2-combining power of the blood and the β-hydroxybutyric acid, since this substance is in major part responsible for decrease in alkaline reserve when the decrease is due to a ketosis (3). It was also desired to observe the relative proportions of acetone and acetoacetic acid and β-hydroxybutyric acid, since it was thought that this would throw some light on the rôle of ether in the production of acetone bodies. The ether contained in the postoperative blood specimens was removed from the filtrate at the end of 30 minutes boiling with the reagents by passing a current of air through the hot liquid for 10 minutes, after the acetone precipitate had been filtered off. This was found by experiment to remove completely all the ether. After cooling, 160 cc. of the fluid were returned to the flask and 5 cc. of 5 per cent dichromate were added after boiling had commenced. The CO_2-combining power was determined by the Van Slyke method (4).

The average of the preoperative values given in Table I, when expressed as total acetone bodies, is about 1 mg. per 100 cc. higher than that stated as the upper normal limit by Van Slyke and Fitz (2); the majority of those for acetoacetic acid and acetone (as acetone) and for β-hydroxybutyric acid are in agreement with those obtained by Marriott (5) for normal blood.[1] The average value for acetoacetic acid and acetone (as acetone) was 0.91 mg., that for β-acid 6.12 mg. per 100 cc. None of these patients was known to be suffering from a ketosis at the time these bloods were taken, the urines of all, except three which were not tested, being found negative to the ferric chloride and sodium nitro-

[1] It should be noted that β-hydroxybutyric acid values in Tables I, II, and III are expressed in terms of β-hydroxybutyric acid, while those given by Marriott are in terms of acetone. Conversion of the β-acid values to terms of acetone may be made by dividing by 2.36.

TABLE I.

Patient.	Sex.	Age.	Preoperative.				Duration of ether administration.	Postoperative.					Remarks.
			Urine	100 cc. of blood.				Urine.		100 cc. of blood.			
			Acetone bodies.*	Acetoacetic acid and acetone (as acetone).	β-hydroxybutyric acid.	CO_2-combining power.		Nature of collection.	Acetone bodies.	Acetoacetic acid and acetone (as acetone).	β-hydroxybutyric acid.	CO_2-combining power.	
				mg.	*mg.*	*per cent*	*min.*		*mg.*	*mg.*	*mg.*	*per cent*	
1. J. R.	♂	25		2.4	8.04		21	1 voiding.		1.0	18.6		Subacute appendicitis. Appendectomy.
2. S. J.	♂	28	Negative.	0.0	3.55		30	1 "	Trace.*	1.2	3.52	47.5	Fistula repaired. Hemorrhoidectomy.
3. E. C.	♂	36	"	2.28	3.55	53.2	26	1 "	Faint trace.*	1.0	12.8	48.5	Hemorrhoidectomy.
4. W. D.	♂	37	"	1.0	4.28	69.2	40			1.0	5.53	59.8	Inoperable carcinoma of stomach.
5. E. C.	♀	32	"	0.6	6.62	51.3	45	24 hrs.	190 (total).	1.8	9.94	44.7	Femoral hernia.
6. E. P.	♂	74		0.4	2.36	53.0	40			0.75	0.0	44.7	Carcinoma of bladder.
7. P. O.	♂	55	Negative.	0.8	4.74		40			2.2	6.63	48.5	Hemorrhoidectomy.
8. M. B.	♀	36	"	0.2	24.6	54.0	70	24 hrs.	264 (total).	0.6	13.4	40.0	Carcinoma of breast.
9. J. D.	♀	77	"	1.4	1.51	58.9	110			2.0	14.15		Breast amputation for carcinoma.
10. W. C.	♂	50		0.8	7.0	62.4	43			0.4	16.6	51.9	Inoperable carcinoma of bladder.
11. M. M.	♀	27	Negative.	0.6	5.2	58.6	47	Hourly for 3 hrs.	See Table II.	0.6	3.55	50.0	Pericholecystic adhesions removed. Appendectomy.
12. M. P.	♂	29	"	0.48	2.0	55.1	77	Every 2 hrs. for 6 hrs.	See Table III.	1.0	4.68	46.2	Gastroenterostomy. Appendectomy. Removal of adhesions.

* Ferric chloride and sodium nitroprusside tests.

The condition of all the above patients was good immediately after operation, and all had an uneventful recovery from the anesthetic.

prusside tests. The average duration of administration of ether for the series was 48 minutes. The average postoperative value for acetoacetic acid and acetone was 1.13 mg., and for β-acid 9.2 mg., per 100 cc. The average preoperative CO_2-combining power was 57.3, postoperative, 48.6 volumes per cent. The average increase of acetoacetic acid and acetone was 0.22 mg., of β-acid 3.08 mg., per 100 cc. In several instances, but particularly in the case of M. B., there was apparently an actual decrease in the acetone bodies following anesthesia. As will be noted, the preoperative β-acid value for M. B. was exceptionally high; unfortunately, enough blood was not available or obtainable for a duplicate determination to check this result. In all cases the β-acid values were affected by the anesthetic to a greater extent than the acetoacetic acid and acetone values, the latter remaining more constant. The most significant rise in the β-acid value was seen in the case of J. D. where anesthesia was prolonged for 110 minutes, but in no case was the increase sufficient immediately after the anesthetic to become dangerous to the patient, as it is said that diabetics under good control show 10 to 40 mg. of acetone bodies (as acetone) per 100 cc. of blood (2). Unfortunately, sufficient blood was not obtained from J. D. following anesthesia for a determination of the CO_2-combining power.

It would not only appear, from the figures in Table I, that the increased values for acetone bodies could have had little to do with the decreased bicarbonate, but it is also somewhat doubtful if the figures obtained on postoperative bloods in all cases represent merely the acetone bodies. It was suspected that, since the blood fat content is increased as a result of ether administration (6), this might become an interfering substance as its solution in the ether of the blood would permit it to escape through the filter during the removal of the precipitated proteins; and, since ether could readily be detected in the filtrate, a few experiments were carried out which confirmed this suspicion. At first, fat shaken with 25 cc. of water (in order to have the usual concentration of reagents) was boiled with the reagents including dichromate for 1½ hours and a precipitate was recovered. Then some fat, of such quantity that, when added to 10 cc. of normal blood, it would approximately double its fat content, was thoroughly mixed with a 10 cc. portion of a blood specimen; and the

same amount of fat in solution in a small quantity of ether was thoroughly mixed with another 10 cc. portion of the same blood specimen. Determinations of total acetone bodies were then carried out in the usual manner, with the result that the specimen containing the ether yielded more than double the amount of precipitate obtained from the other. Since ether itself forms a precipitate when boiled with the reagents in the presence of dichromate, and since in this experiment the ether was not removed by aeration as previously described, the experiment was repeated, adding the same amount of fat, the fluid being aerated after 30 minutes boiling without dichromate to precipitate the preformed acetoacetic acid and acetone present in the blood. From the specimen to which fat alone was added, no increase in precipitate was obtained over that yielded by the blood alone, the figures being nearly identical. The precipitate from that to which both fat and ether were added, however, was over twelve times as great.

Following this work two series of determinations of acetone bodies, CO_2-combining power, and total fat were made on the bloods of two patients before and after ether anesthesia. The fat determinations were carried out by Bloor's nephelometric method (7). From the first patient blood and urine specimens were collected simultaneously at hourly intervals for 3 hours, and from the second at 2 hour intervals for 6 hours. The urines were likewise analyzed for acetone bodies. The results are expressed in Tables II and III.

The figures in Table II express rather clearly the time necessary for any significant increase in concentration of blood acetone bodies. At 3.00 p.m., immediately after the cessation of 47 minutes of ether administration, there was no increase. At 4.00 p.m. there was apparently an increase in preformed acetoacetic acid and acetone, but still no increase in the β-acid. At 5.00 and at 6.00 p.m., however, the β-acid determinations showed a marked and distinct increase, rising to 6.63 and 10.4 mg., the acetoacetic acid and acetone value remaining about constant around 1 mg. This increase is confirmed by the increased output in the urine where there was a gradual increase for each hourly period coincident with the increased blood concentration. (In examining the table it should be noted that the first urine collection, at

4.00 p.m., was for a period of 2 hours, the bladder being emptied at 2.00, and no collection being made at 3.00.)

In connection with the results of these determinations, it is now interesting to note what happened to the plasma CO_2-combining powers for the same periods. Before anesthesia the figure obtained was 58.6; immediately after, it had fallen to 50.0; one hour after this, it had risen slightly to 52.8, and at the end of another hour it again had decreased to 50.0, coincident with an

TABLE II.

Determination of Acetone Bodies, CO_2-Combining Power, and Total Fat.

M. M., ♀, aged 27. Pericholecystic adhesions removed. Appendectomy.

Time.	Urine.			100 cc. of blood.				Remarks.
	Volume.	* Acetoacetic acid and acetone (as acetone).	β-hydroxybutyric acid.	Acetoacetic acid and acetone (as acetone).	β-hydroxybutyric acid.	Total fat.	CO_2-combining power.	
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*per cent*	
2.00 p.m.		Negative.*		0.6	5.2	1.39	58.6	Bladder emptied.
2.13 "								Ether anesthesia begun.
3.00 "				0.6	3.55	1.51	50.0	Administration of ether ended.
4.00 "	57	1.37	2.87	1.2	2.84	1.47	52.8	
5.00 "	22	0.88	2.7	1.0	6.63	2.11	50.0	Slight hemolysis.
6.00 "	26	0.93	6.27	1.4	10.4	2.02	54.8	

* Ferric chloride and sodium nitroprusside tests.

Each of the postoperative blood specimens gave an odor of ether. Postoperative urines were withdrawn by catheter.

increase in β-acid from 2.84 mg. for the hour previous to 6.63 mg. It was thought, however, that this slight decrease was probably due to a slight hemolysis which was present in this specimen, but was not present in the others. The final determination is most significant, however, since there was a distinct increase in CO_2-combining power, beyond any possible limit of error, to 54.8 coincident with an increase in β-acid from 6.63 to 10.4 mg.

Figures in Table III are in harmony with those in Table II, though they are not quite so striking. Urine figures show the same gradual increase. (The 3.45 p.m. collection of 243 cc. was for $1\frac{3}{4}$ hours.) Unfortunately three of the blood acetone body determinations were accidentally spoiled. As a result of the anesthetic, the CO_2-combining power decreased from 55.1 to 46.2, coincident with an increase in the figures for acetoacetic acid and acetone from 0.48 to 1.0 mg., and for β-acid from 2.0 to

TABLE III.

Determination of Acetone Bodies, CO_2-Combining Power, and Total Fat.

M. P., ♂, aged 29. Posterior gastroenterostomy. Separation of adhesions. Appendectomy.

Time.	Urine.			100 cc. of blood.				Remarks.
	Volume.	Acetoacetic acid and acetone (as acetone).	β-hydroxybutyric acid.	Acetoacetic acid and acetone (as acetone).	β-hydroxybutyric acid.	Total fat.	CO_2-combining power.	
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*per cent*	
2.00 p.m.		Negative.*		0.48	2.0	0.653	55.1	Bladder emptied. Ether anesthesia begun.
3.17 "								Administration of ether ended.
3.45 "	243	0.0	9.77	1.0	4.64	0.629	46.2	
5.45 "	40	1.14	9.25		7.04	0.707	51.0	
7.45 "	33	2.18	6.64	0.6	6.04	0.725	51.0	
9.45 "	50	2.84	11.0			0.666	50.8	

* Ferric chloride and sodium nitroprusside tests.
Postoperative urines were withdrawn by catheter.

4.64 mg. The most significant feature, however, is the increase in CO_2-combining power from 46.2 to 51.0 during the next period of 2 hours coincident with an increase in β-acid from 4.64 to 7.04 mg.

Blood fat determinations were carried out in the last two cases because it was thought that, since traces of fat may escape through the filter during the removal of the proteins and appear in the filtrate, this might account in part for the increased value obtained

following ether anesthesia. Very probably this is true, and many figures in Table I are undoubtedly higher than they should be, for the reason that the blood withdrawn immediately after anesthesia contained relatively large amounts of ether. This could not be aerated off before precipitation of the blood proteins or before boiling the filtrate with the reagents because of the probability of volatilizing the preformed acetone at the same time (8). The β-acid values expressed in Tables II and III, however, show definite, true increases in this substance, because the fat values remained constant while the β-acid values increased; the ether was becoming less concentrated in the later specimens; and, most important, corresponding increases in excretion were found in the urine. It would appear, therefore, from the results as a whole, that the blood acetone bodies could have had little to do with the decreased plasma bicarbonate after ether anesthesia. This decrease was undoubtedly due to other factors (9).

EXPERIMENTAL.

Interfering Substances.

1. Ether.—Ether was boiled with the reagents to determine whether or not a precipitate was formed from it. A precipitate formed only after the addition of 5 cc. of 5 per cent dichromate, the fluid becoming green in color. It was observed in this experiment that some ether was being lost through the reflux condenser.

2. Fat.—(*A*) Neutral olive oil was boiled with the reagents in usual concentration to determine whether or not this would give a precipitate, with the result that a precipitate formed in considerable quantity after dichromate was added. This was freed from excess fat by extraction with alcohol and ether.

(*B*) 60 mg. of neutral olive oil were thoroughly mixed with a 10 cc. portion of a blood specimen. A determination for total acetone bodies was then carried out on this specimen with the result that, when calculated per 100 cc. of blood, a value of 6.6 mg. was obtained. At the same time a solution of 60 mg. of olive oil in a little ether (about 1 or 2 cc.) was thoroughly mixed with another 10 cc. portion of the same blood. A determination for total acetone bodies was then carried out with the result that 15.8 mg. (as acetone) were obtained (calculated per 100 cc. of blood).

(*C*) 10 cc. of a blood specimen were analyzed for total acetone bodies in the usual manner. The result was 0.59 mg. as acetone per 100 cc. of blood. To another 10 cc. portion of the same blood specimen 60 mg. of olive oil (about equal to the normal fat content of 10 cc. of blood) were added, and an analysis was made as with the first portion. 0.51 mg. per 100 cc. of blood was obtained. To a third portion of the same blood specimen were added 60 mg. of olive oil in solution in a small amount of ether, and the whole was thoroughly mixed. An analysis was then made for total acetone bodies, the filtrate first being boiled with the H_2SO_4 and $HgSO_4$ reagents, but without dichromate for 30 minutes, to precipitate the preformed acetone. The hot liquid was then aerated for 10 minutes to remove the ether; 5 cc. of 5 per cent dichromate were added after boiling had commenced, and after boiling had continued for 1½ hours longer the determination was completed. The result was 7.42 mg., as acetone, calculated per 100 cc. of blood, over twelve times the result obtained from analysis of the blood alone.

3. Removal versus Non-Removal of Ether from Blood Filtrate.—Total acetone bodies were determined on a specimen of blood, withdrawn immediately after ether anesthesia, having a strong odor of ether. The ether was removed by aeration after the first 30 minutes of boiling, as previously described. The result was 9.4 mg., as acetone, per 100 cc. of blood. A simultaneous determination of total acetone bodies was made on the same blood, no attempt being made to remove the ether. 10.0 mg., as acetone, per 100 cc. of blood were obtained.

4. Glycerol.—A few drops of glycerol, weighing exactly 129 mg., were placed in a 500 cc. Erlenmeyer flask with 25 cc. of water. Procedure was then carried out as for total acetone bodies. Upon the addition of the dichromate this was immediately reduced giving a green color. A precipitate of 462.2 mg. was formed which was only slightly soluble in HCl and gave a positive Lieben's, but a negative Gunning's, iodoform test.

5. Oleic Acid.—Oleic acid was treated in the same manner as the glycerol, 115.6 mg. forming 12.4 mg. of precipitate with the reagents.

Tests of the reagents showed that these alone formed no precipitate. No blank determinations were made on any of the blood or urine filtrates.

SUMMARY.

Observations of the acetoacetic acid and acetone (expressed as acetone) and the β-hydroxybutyric acid of the blood were made before and after ether anesthesia on twelve patients by the method of Van Slyke and Fitz, and the CO_2-combining powers before and after anesthesia on eight of these patients by the Van Slyke method. A series of blood and urine specimens from each of two of these patients was also withdrawn over a number of hours following ether anesthesia, the blood, in addition to the above mentioned determinations, being examined for total fats by Bloor's method, and the urine for acetoacetic acid and acetone (as acetone) and β-hydroxybutyric acid by Van Slyke's method. A number of experiments were carried out to determine sources of error in analyzing blood containing ether. Removal of ether from blood filtrates was accomplished by aeration of the mixed filtrate and reagents after 30 minutes boiling to precipitate the preformed acetone and acetoacetic acid.

Contrary to the results of Reimann and Bloom, it was found that the blood concentration of acetone bodies was but little affected during the period of anesthesia, which was, on the average, a period of 48 minutes. It was found in the two cases examined over a longer period that there was an increase in these substances a few hours later, a finding which was confirmed by their increased output in the urine. The CO_2-combining powers, however, increased even during increase of β-hydroxybutyric acid.

The results obtained for acetone bodies on bloods taken previous to anesthesia were, on the average, lower than those reported by Reimann and Bloom, the majority agreeing with the figures given by Van Slyke and Fitz, and also by Marriott, for normal blood. It is possible that Reimann and Bloom's high results find explanation in occasional failure to filter off their precipitate soon after the period of boiling was finished. Van Slyke and Fitz (10) have, since the appearance of Reimann and Bloom's paper, pointed out that this precaution is necessary.

Experimental data indicate that fat in the presence of ether and ether itself may interfere with the accuracy of the method, since both may form a precipitate with the reagents. It could not be shown from analyses on postoperative bloods that ether

directly effected much error, but through its ability to penetrate the filter it apparently carried with it some fat in solution causing an increase in precipitate. Glycerol formed a comparatively large precipitate with the reagents, oleic acid a much smaller precipitate.

CONCLUSIONS.

Acetone bodies were not formed promptly enough during ether anesthesia in the cases reported to account for the decreased plasma bicarbonate.

Analyses reported for β-hydroxybutyric acid on postoperative bloods may in some instances have been too high due to an error introduced as a result of the ether content.

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MILK AS A SOURCE OF WATER-SOLUBLE VITAMINE. II.*

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(Received for publication, February 26, 1920.)

Early in the course of our nutrition experiments upon rats[1] we learned that both milk—dried milk powder was used—and what we termed "protein-free milk" prepared from it furnished something without which the animals could not grow satisfactorily when they were kept on "synthetic" diets consisting of mixtures of more or less purified isolated food substances. Subsequently Hopkins[2] published striking experiments showing that fresh milk, used in quantities surprisingly small when compared to the amounts which we had incorporated into our successful rat diets, also was effective in securing growth on the so called synthetic food mixtures consisting of purified proteins, fat, carbohydrates, and salts. Experience had shown us the necessity of incorporating as much as 28 per cent of our protein-free milk into our food mixture in order to secure adequate growth. The actual milk solids furnished by some of the quantities of fresh milk which Hopkins used effectively amounted "to no more than from 1 to 3 or 4% of the whole food eaten." The calorific value of Hopkins' foods was comparable with that fed by us.

Since these pioneer experiments our knowledge of the significance of those nutrition-promoting substances now commonly

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911.

[2] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

designated as vitamines has been largely augmented. Bearing this in mind we undertook further investigations in the hope of explaining the apparent discrepancies between our earlier results and those of Hopkins as to the minimal amount of milk needed to supply an adequate quota of the water-soluble vitamine. The entire subject has already been reviewed in some detail by us.[3]

Attempting to duplicate the results of Hopkins we fed diets consisting of casein or edestin, starch, a salt mixture, lard, and butter fat along with fresh milk offered in varying quantities. In contradiction to some of Hopkins' results we found, under the conditions of our investigation, that 2 cc. of milk per day rarely sufficed to enable rats, on the diets mentioned, to make more than very slight gains in weight. Many of the animals were barely maintained when such small quantities furnished the sole source of water-soluble vitamine. Not until at least 16 cc. of fresh milk per day were supplied along with the food mixture was anything approaching a normal rate of growth secured. Even this amount sometimes failed.

The large quantity of fresh milk thus indicated as necessary to supply water-soluble vitamine is equivalent, broadly speaking, to the quantities of protein-free milk which were found necessary, in our earlier experiments, to secure adequate growth with the synthetic dietaries, even when due attention was paid to the calorific make-up of the food, the quality and content of its protein, and the supply of fat-soluble vitamine. As was pointed out in the previous report of our investigation we tested the possibility that pasteurization of milk, as it is conducted preliminary to the distribution of most city milk supplies, might affect the vitamine content of the milk employed by us. The use of unpasteurized milk of known origin did not improve our results; the smaller quantities—less than 16 cc.—still proved inadequate to promote growth at a maximum rate.

To another aspect of this question we have referred as follows:[3]

"That the deficiency of diets containing the lesser amounts of milk involves the vitamine factor is rendered more than probable by the fact that these comparatively small additions of yeast, the highly efficient growth-promoting power of which we have discussed elsewhere, sufficed

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537.

to render the previously inadequate ration satisfactory for growth. As a rule the most significant outcome of the yeast additions (which were fed apart from the rest of the food and therefore could not have altered its flavor) was a larger food intake."

Our earlier investigations with fresh milk were carried out during the winter season when the cows in this region are deprived of green pasture and are stall-fed. It seemed not impossible, therefore, that the relatively large quantity of milk necessitated as a source of water-soluble vitamine in those experiments might be associated with quality of milk inferior from the vitamine standpoint, owing to the winter diet of the cows. Other investigators have of late intimated that the content of the milk in various nutrition-promoting factors may be markedly altered by the character of the diet from which the various vitamines are assumed to be derived. Our own investigations have taught us that the grasses and other green foods, which cattle are likely to obtain in greater abundance during the summer season, are rich in vitamines.[4]

Consequently we have undertaken a further series of experiments on rats which were supplied with a diet presumably adequate in every respect except for the absence of the water-soluble vitamine.[5] The food mixtures consisted of:

	per cent
Casein	18
Salt mixture*	4
Starch	49
Butter fat	9
Lard	20

* The composition of the salt mixture used is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

In addition to this mixture, which was fed *ad libitum*, the rats received daily, in measured portions in a separate container,

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; xxxix, 29.

[5] In numerous experiments with such a food mixture we have demonstrated that the addition of 0.2 gm. of dried brewery yeast fed apart from the ration suffices to induce the animals to eat sufficient food and promote growth, whereas without the yeast addendum their food intake gradually declines and they die within a period of 40 to 80 days (Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35).

varying quantities of unpasteurized milk obtained fresh from cows known to be feeding in open pasture. Until within the last month of our experiments the five cows were kept all day in the pasture. During the last month, owing to the lateness of the season, the available pasturage was small. Night and morning the cows were fed with rations of corn gluten and wheat bran together with hay and corn stalks. This method of feeding is the one usually employed in this region where milch cows are pasture-fed during the summer.

The outcome has been comparable with the experiments earlier published, as the appended charts show. Thus with additions of 2 cc. of summer milk (Rats 5904, 5911, 5891, Chart I), fed during a period of 37 or more days, no permanent gains were secured. Additions of 5 cc. (see also Rats 5944, 5943, 5947, Chart II) invariably produced better, though by no means adequate, growth; nor was the latter usually obtained with daily additions of 10 cc. of summer milk. Whenever the vitamine supplement in the form of milk was still further increased, improvement in the rate of growth occurred. Of course, the rats ate more in these cases. The weekly food intakes, not including that furnished by the milk solids, are shown in the charts.

The inferiority of even 15 cc. of the fresh, unpasteurized summer milk as a source of water-soluble vitamine, in contrast with 0.2 gm. of dried brewery yeast, is indicated by the more rapid gains made by all the animals thus tested when the yeast addendum replaced the milk. In our previous report we have indicated that this is not due to the large volume of fluid intake, represented by 15 cc. of milk, preventing the ingestion of a sufficient amount of the basal diet; for in the experiments there recorded, when yeast was furnished in addition to both the large volume of milk and the basal diet, increased intakes of food with additional gains in weight followed.

When the experiments were *started* with 10 cc. of the summer milk, there was no greater advantage (see Chart III, Rats 6096, 6097, 6101); and even 15 cc. of milk fed from the start (Rats 6093, 6098, 6092, Chart III) barely sufficed as a source of water-soluble vitamine to promote growth at a normal rate. Here again an inspection of the charts shows the superiority of 0.2 gm. of dried yeast, in contrast with the large volumes of milk.

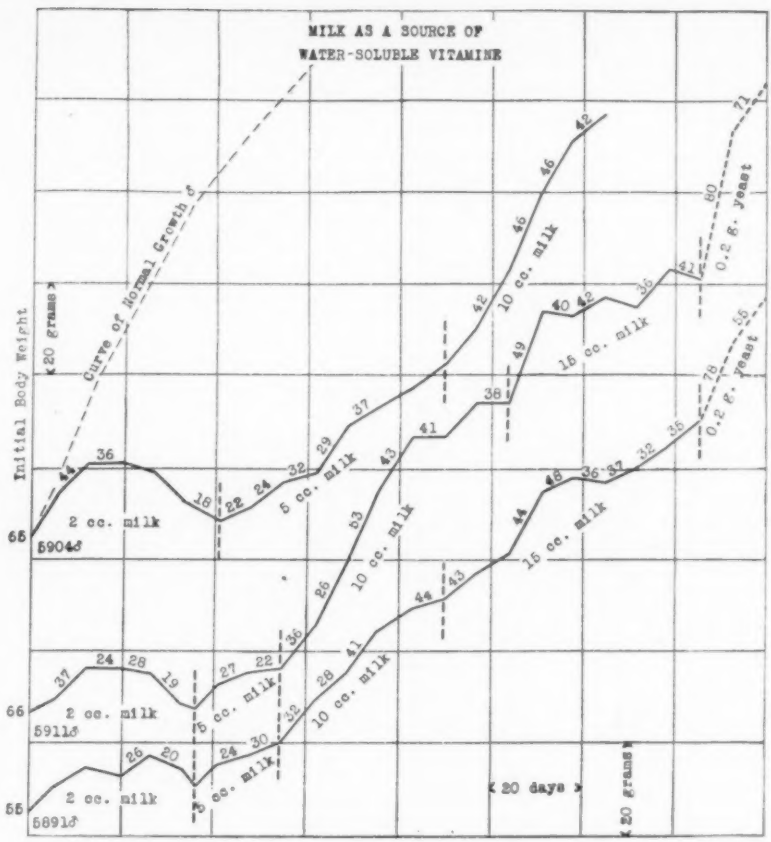

CHART I. Showing the effect upon growing white rats of increasing quantities of fresh unpasteurized summer cow's milk, fed in addition to and apart from a standard diet devoid of water-soluble vitamine. The composition of the food mixture is given on page 517. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth. The interrupted line (Rats 5891 and 5911) indicates the superiority of small quantities of brewers' yeast in comparison with large volumes of milk as a source of water-soluble vitamine, and also the adequacy of the ration apart from its content of the latter.

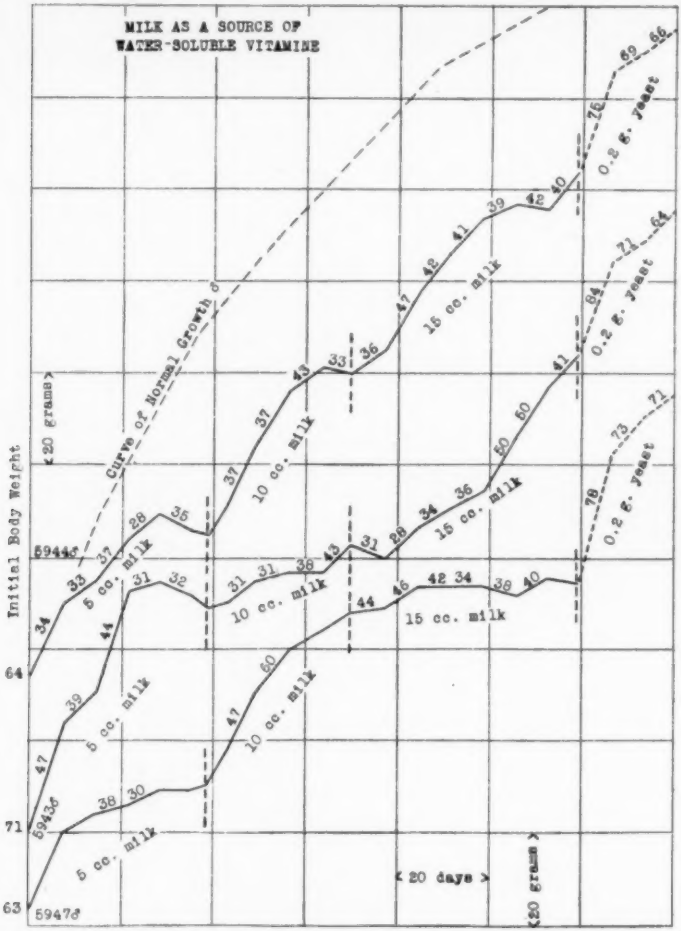

Chart II. Supplementing Chart I in showing the effect upon growing white rats of increasing quantities of fresh unpasteurized summer cow's milk, fed in addition to and apart from a standard diet devoid of water-soluble vitamine. The composition of the food mixture is given on page 517. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth. The interrupted line (Rats 5944, 5943, 5947) indicates the superiority of small quantities of brewers' yeast in comparison with large volumes of milk as a source of water-soluble vitamine, and also the adequacy of the ration apart from its content of the latter.

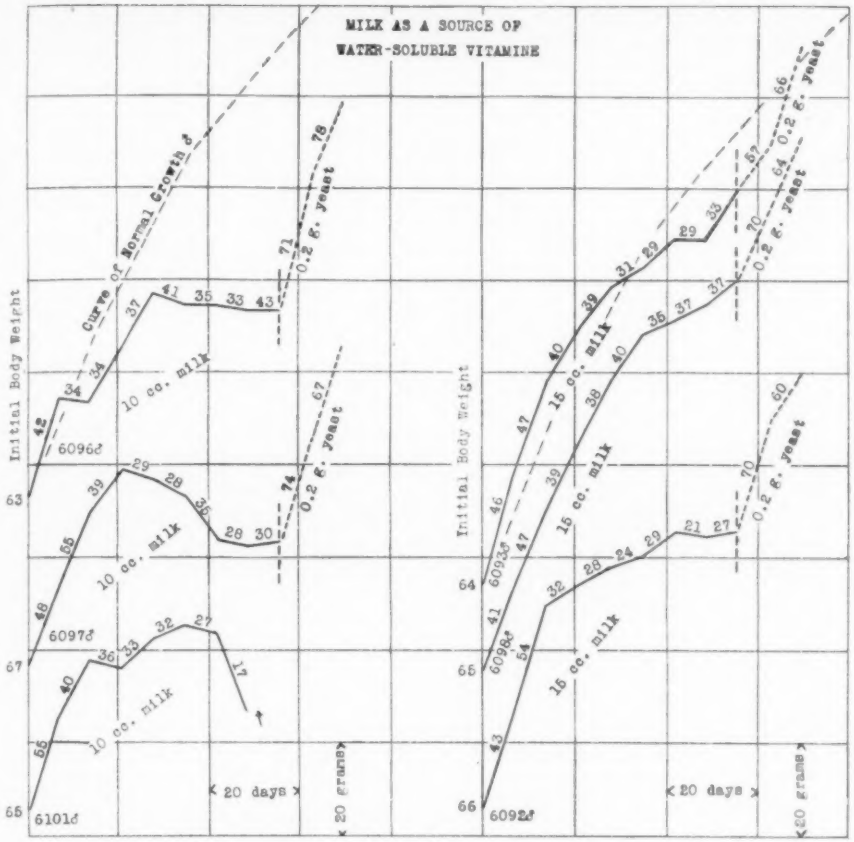

CHART III. Showing the failure of 10 cc. of fresh unpasteurized summer cow's milk (Rats 6096, 6097, 6101), fed apart from and in addition to a ration adequate except in respect to the lack of water-soluble vitamine, to promote growth at a normal rate. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth. The prompt increase in food intake and growth response (shown by the interrupted line) when milk was replaced by small daily doses of brewers' yeast indicate that the previous failures were associated with the lack of water-soluble vitamine.

When 15 cc. of the same milk (Rats 6093, 6098, 6092) supplied the water-soluble vitamine better growth responses were obtained; but not so good as those secured by the feeding of 0.2 gm. of dried yeast.

In the original experiments of Hopkins the food was reported to have the following composition:[2]

	Pure casein mixture. *per cent*	"Protene" mixture. *per cent*
Protein	22.0	21.3
Starch	42.0	42.0
Cane Sugar	21.0	21.0
Lard	12.4	12.4
Salts*	2.6	3.3

* "The salts added were obtained by incinerating the normal laboratory food on which the rats had been kept when not under experiment, and consisted of equal parts of the ash of oats and dog-biscuits."

It was, of course, possible that the ash of oats and dog biscuits there used might contain some unsuspected inorganic ingredients which would account for the remarkable gains claimed by Hopkins for some of the animals to which the small quantities of milk were supplied. We therefore imitated the diets used by him as closely as the preparation of ash from a product of such uncertain composition as dog bread would permit. These food mixtures consisted of:

	per cent
Casein	18.0
Salts*	4.5
Starch	50.5
Butter fat	9.0
Lard	18.0

* The salts consisted of equal parts of the ash of dog bread and the ash of whole oats.

The outcome was in harmony with all our experience in showing that even additions of 10 cc. of fresh milk per day were insufficient to effect a food intake adequate for growth at a normal rate. Chart IV, showing the results in graphic form, also includes the growth curves of rats fed on diets containing our own often described salt mixture. The milk in the two series was obtained from the same source, so that the experiments are strictly comparable. The outcomes are not essentially different.

Recent studies of the antiscorbutic value of cow's milk[6] have indicated that on this score it must be classed as less valuable than many of the raw fruits and vegetables. Whereas quantities of the latter—less than 10 gm. daily—will prevent scurvy in guinea pigs upon a diet otherwise devoid of antiscorbutic material, 100 to 150 cc. daily of raw cow's milk are required for this species, according to Barnes and Hume; while monkeys require larger quantities. Similarly, relatively large

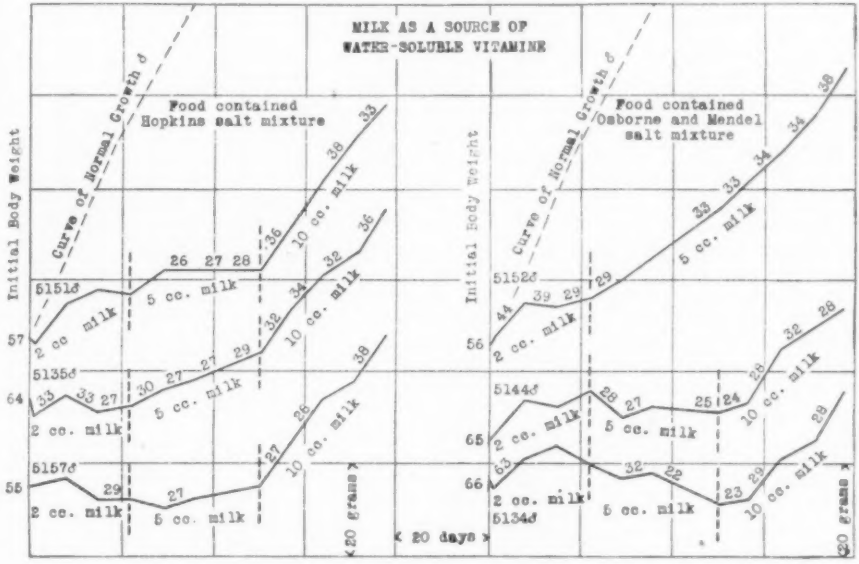

CHART IV. Showing that rats grow at approximately the same rate whether they receive a salt mixture similar to that used by Hopkins or the salt mixture used by ourselves, when like quantities of milk supply all the water-soluble vitamine. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth.

quantities of milk are required to produce the increased intake of food and improved rate of growth, which are readily secured by very small quantities of many (dried) green vegetables. The consequences of this relative poverty of milk in water-soluble vitamine for the artificial feeding of infants have already been referred to by us.

[6] Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425. Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131; *Lancet*, 1918, i, 1. Barnes, R. E., and Hume, E. M., *Lancet*, 1919, ii, 323.

THE EFFECT OF INTRAVENOUS INJECTIONS OF ACTIVE DEPOSIT OF RADIUM ON METABOLISM IN THE DOG.

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There have been few experiments reported on the effect of radium on normal metabolism. Berg and Welker (1) have studied the effect of radium salts upon the metabolism of dogs. The doses employed were very small, but these investigators concluded that ingestion of radium *per os* was followed by a stimulation of the catabolic processes as indicated by a slightly increased output of nitrogen in the urine. An increased volume of urine was also noted.

In experiments where radium salt is given, it is obvious that one cannot be certain whether the effects noted are due to the element radium or the rays emitted by the element.

The present work has been planned to study the effect of the active rays upon the general metabolism of the dog. Solutions of sodium chloride which contained active deposit from radium emanation were used. To prepare the solutions, sodium chloride is first dried and packed into a bulb and left in contact with radium emanation for 3 or 4 hours.[1] The emanation is then pumped off and the salt dissolved in water in such concentration as to give a solution isotonic with the blood. The solution is drawn into a syringe and the amount of activity determined by a γ-ray instrument. After the injection, the radioactivity remaining in the syringe is again determined and the amount injected computed by difference. The radioactivity is expressed in millicuries. It should be noted that the number of millicuries

[1] The preparation of the radioactive sodium chloride was first called to our attention by Dr. William Duane, of Harvard University.

injected cannot be controlled exactly as an irregular quantity (25 to 50 per cent) will remain in the syringe. The rapid decay of the active deposit introduces a second source of error in estimating the quantity injected. Radium A decays completely within 15 minutes after the preparation of the solutions. Since our injections were made after 15 minutes, only Radium C need be taken into account. Radium C falls to 3 per cent of its initial value within 3 hours, so that the physiological activity of the injected solution can last only a relatively short time. The physiological effects are presumably due to the α-radiation.

EXPERIMENTAL.

Two animals were employed in the experiments described below. Dog 1 was a Dalmatian female of about 15 kilos. Benedict (2) has shown that this breed of dog regularly excretes large amounts of uric acid. We therefore used a Dalmatian in order to bring out any possible effect of the emanation upon the metabolism of this substance. Dog 2 was a bull-terrier female of about 12 kilos. Each animal was kept in a metal metabolism cage constructed for the proper separation and collection of urine and feces.

The diets employed for both dogs were made up of cracker meal, dog biscuit, evaporated milk, bone ash, and (in the case of Dog 2) a little casein. Dog 1 received 6 gm. of nitrogen and about 1,000 calories per day; Dog 2 received 5 gm. of nitrogen and about 660 calories per day.

The animals were catheterized daily just before feeding, and the bladder was washed out with distilled water followed by a little boric acid. The urine was collected under toluene and usually analyzed on the same day. The rectal temperature was taken and blood counts were made at frequent intervals. The injections were made into one of the superficial veins of the ear.

All analyses were made in duplicate; nitrogen was determined by the Kjeldahl-Gunning method, urea by a macro-urease method, ammonia by the older macro method of Folin. Creatinine was determined by the colorimeter method of Folin, uric acid by the Benedict-Hitchcock modification of the Folin-Denis method, and phosphates were determined by titration with uranium acetate. Albumin, sugar, and creatine were tested for frequently with negative results.

Experiment 1.

Table I records the experiments with the Dalmatian dog. The first 6 days (February 28 to March 5) serve as the control period before the first injection. On March 5 the dog received an intra-

venous injection of 95 millicuries of the active deposit. On the day of the injection there seemed to be no effects on the general condition of the animal but on the 2 following days the dog had to be coaxed to eat the food and the feces were softer than usual. After this no further general effects were present. An examination of the nitrogen partition shows a sharp rise in the total nitrogen output, which reaches its maximum on the 2nd day after the injection. This increased nitrogen is distributed among all the nitrogen constituents except creatinine. Ammonia increases proportionately more than does urea. Indeed the increase in the ammonia suggests a definite acidosis. The uric acid increases by about 50 per cent over that of the preliminary period. It is possible that this is associated with the destruction of white blood corpuscles which follows the injection. Phosphates and urinary volume both tend to show definite increases over the preliminary period.

The total nitrogen remains high for 5 days after the injection, and then drops suddenly. The ammonia and particularly the uric acid drop more slowly. The latter does not reach the level of the preliminary period until 12 days after the injection.

On April 4 the dog received a second intravenous injection of 30 millicuries of the active deposit. This dose is scarcely a third of the quantity given in the preceding experiment. The total nitrogen again increases, and again the maximal figure is reached on the 2nd day after the injection. The small dose employed in this experiment is followed by only a slight and transitory rise in uric acid. On the 3rd and 4th days after the injection creatinine shows an increase well above the preliminary period.

On April 15 the dog received its third injection of the active deposit. On this day 42 millicuries were given. This injection is followed by a marked and prolonged effect. Total nitrogen, urea, and ammonia increase and remain high for almost 2 weeks. Creatinine and uric acid both show a marked increase. The high figures for those constituents continue for about 8 days after the injection. Throughout the series of treatments on Dog 1, we find a steady increase in the volume of urine eliminated. At first the amount was 200 cc. and after the treatments it gradually rose to 1 liter.

TABLE I.

Period.	Date.	Weight of dog.	Urine.											Titratable 0.1 N acid.	Temperature.	Remarks.
			Volume.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Creatinine N.		Uric acid N.		P_2O_5		
	1919	kg.	cc.		gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	cc.	°F.	
Control.	Feb. 28		210	1.028	4.21	3.58	85.00	0.241	5.73	0.094	2.23	0.101	2.40			
	Mar. 1		242	1.027	4.89	3.98	81.49	0.261	5.34	0.093	1.90	0.101	2.08			
	" 2–3		576	1.021	4.60	3.85	83.78	0.241	5.24	0.089	1.93	0.093	2.02	0.737		
	" 4		480	1.016	4.83	4.13	85.60	0.219	4.53	0.096	1.98	0.094	1.95	1.05		
I*	" 5		210	1.030	4.55	3.84	84.30	0.250	5.49	0.098	2.15	0.101	2.23	1.00		Red cells 5,000,000 White " 11,640 Hemoglobin 85 %
	" 6		390	1.025	5.26	4.51	85.84	0.236	4.49	0.096	1.82	0.130	2.48	1.22		Red cells 4,400,000 White " 10,250 Hemoglobin 82%
	" 7	14.77	590	1.016	5.43	4.56	83.91	0.351	6.51	0.097	1.79	0.155	2.85	1.11		Red cells 4,120,000 White " 8,400 Red " 4,256,000 White " 7,450 Hemoglobin 70%
	" 8		510	1.018	5.21	4.41	84.74	0.331	6.36	0.098	1.84	0.135	2.60	0.902		
	" 9		390	1.019	5.14	4.44	86.40	0.304	5.91	0.094	1.83	0.159	3.10	0.853		
	" 10		348	1.022	5.13	4.43	86.60	0.255	4.99	0.094	1.84	0.159	3.11	0.728		
	" 11	14.88	327	1.020	4.51	3.83	85.00	0.281	6.23	0.094	2.09	0.125	2.77	0.894		
	" 12	15.00	395	1.019	4.65	3.82	82.15	0.253	5.44	0.098	2.10	0.113	2.48	0.759		
	" 13	14.88	490	1.018	4.73	4.02	84.90	0.222	4.69	0.101	2.14	0.122	2.57	0.780		
	" 14	14.77	392	1.020	4.92	4.07	82.57	0.269	5.47	0.099	1.99	0.126	2.56	0.863		
	" 15		438	1.017	4.55	3.85	84.69	0.247	5.43	0.095	2.10	0.113	2.49	0.749		Red cells 4,600,000 White " 4,800 Red " 4,640,000 White " 6,900

	Mar. 16	15.00	450	1.018	4.65	3.99	85.90	0.213	4.59	0.094	2.02	0.114	2.45	0.759			
	" 31		295	1.020	4.24	3.58	84.38	0.255	6.02	0.094	2.22	0.098	2.31	0.790	84		
	Apr. 1		410	1.023	4.59	3.91	85.12	0.247	5.38	0.097	2.13	0.109	2.38	0.873	100		
	" 2	15.11	345	1.021	4.86	4.17	85.76	0.218	4.49	0.100	2.06	0.102	2.10	0.811	76		
	" 3		410	1.016	4.66	4.02	86.50	0.235	5.06	0.098	2.11	0.095	2.05	0.852	104		
II†	" 4		395	1.016	4.86	4.16	85.75	0.216	4.45	0.101	2.08	0.111	2.30	0.811	80	101.2	
	" 5		508	1.015	4.89	4.23	86.68	0.202	4.13	0.099	2.03	0.107	2.20	0.873	84		
	" 6		430	1.019	5.04	4.19	83.20	0.261	5.18	0.118	2.34	0.101	2.00	0.998	92	99.8	
	" 7		325	1.018	4.77	3.99	83.53	0.196	4.11	0.121	2.54	0.101	2.13	0.831	76		Red cells 4,136,000 White " 10,200 Hemoglobin 80%
	" 8		518	1.014	4.66	4.02	86.30	0.215	4.62	0.103	2.22	0.095	2.04	0.915	96	99.4	
	" 9	15.30	519	1.016	5.18	4.33	83.64	0.215	4.16	0.120	2.31	0.100	1.95	0.831	52	99.3	
	" 10		500	1.014	4.72	3.88	82.34	0.252	5.35	0.122	2.60	0.099	2.10	0.894	100		
	" 11	15.30	490	1.014	4.97	4.13	83.08	0.252	5.08	0.125	2.52	0.106	2.13	0.831	80	100.4	
	" 12		382	1.016	4.63	3.93	84.90	0.235	5.09	0.124	2.68	0.113	2.44	0.790	80		
	" 13–14	15.5	670	1.020	4.74	4.09	86.20	0.202	4.26	0.124	2.61	0.109	2.31	0.831	80	100.6	Red cells 4,690,000 White " 7,600 Hemoglobin 80%
III‡	" 15		465	1.015	4.52	3.75	82.86	0.241	5.34	0.126	2.80	0.113	2.51	0.749	88		
	" 16		550	1.017	5.24	4.40	83.99	0.207	3.96	0.152	2.90	0.164	3.14	0.852	72	101.0	Red cells 4,890,000
																100.6	White " 6,150
																	Hemoglobin 80%
	" 17	15.2			5.46	4.64	84.61	0.311	5.54	0.169	3.08	0.174	3.17	1.020	96	100.3	
																101.0	
	" 18	15.4	535	1.015	5.17	4.30	83.14	0.280	5.43	0.160	3.10	0.172	3.33	0.831	80	101.4	
																100.7	

* Injected 95 millicuries of active deposit at 1.00 p.m.
† " 30 " " " " 2.40 "
‡ " 42 " " " " 6.30 "

TABLE I—*Concluded.*

Period.	Date.	Weight of dog.	Urine.													Titratable 0.1 N acid.	Temperature.	Remarks.
			Volume.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Creatinine N.		Uric acid N.		P_2O_5				
	1919	*kg.*	*cc.*		*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*		*cc.*	*°F.*	
	Apr. 19	15.2	678	1.012	5.05	4.24	83.89	0.252	5.00	0.159	2.97	0.161	3.19	0.811		60	100.8	
	" 20		570	1.010	4.91	4.13	84.13	0.275	5.60	0.150	3.06	0.151	3.08	0.811			101.4	
	" 21	15.4	750	1.012	5.40	4.63	85.67	0.255	4.72	0.155	2.87	0.129	2.39	0.790		60	101.1	
	" 22		640	1.011	4.85	4.17	85.93	0.287	5.92	0.151	3.04	0.133	2.76	0.790		64	100.7	
	" 23	14.7	670	1.013	5.39	4.58	84.90	0.224	4.16	0.144	2.68	0.124	2.30	0.852		64	100.7	
	" 28		485	1.017	5.42	4.55	83.99	0.264	4.87	0.120	2.22	0.104	1.93	0.790		96	100.6	
	" 29	15.3	600	1.011	5.19	4.48	86.18	0.255	4.92	0.103	1.99	0.104	2.00	0.811		60		
	" 30		848	1.009	5.08	4.39	86.36	0.230	4.53	0.101	1.99	0.111	2.19	0.706		64		
	May 1	15.3	848	1.011	5.42	4.66	85.93	0.233	4.29	0.108	2.00	0.112	2.08	0.786		75	99.3	
	" 2		830	1.010	5.67	4.87	86.95	0.207	3.66	0.109	1.93	0.106	1.87	0.748		72	100.4	
	" 3		850	1.008	4.98	4.26	85.50	0.238	4.90	0.112	2.32	0.127	2.55	0.764		67	100.0	
	" 7	15.5	1,004	1.009	4.91	4.22	85.87	0.198	4.04	0.117	2.39	0.125	2.55	0.786		73	100.5	
IV§	" 8		1,005	1.010	4.86	4.23	86.98	0.188	3.87	0.114	2.36	0.127	2.61	0.786		63	100.4	
																	102.0	

§ Injected 64.5 millicuries of active deposit at 1.19 p.m.

On May 8 the animal received a fourth injection of 64 millicuries. This was followed by vomiting and refusal of most of the food for several days. The fact that the third and fourth injections were smaller in quantity than the first, and were followed by marked general effects, shows that the animal failed to recover completely from previous treatments with the emanation. Shortly after the fourth injection the animal was killed and an autopsy made. The autopsy findings will be reported in detail elsewhere.

Experiment 2.

Table II gives the results of injections of active deposit on the bull terrier; uric acid is eliminated only in very minute quantities, hence quantitative determinations of this constituent have been omitted. After a control period of 5 days, Dog 2 received the first injection of 120 millicuries. Urine voided voluntarily 15 minutes after the injection contained 9 millicuries of radioactivity. A decided rise in total nitrogen was noted which reached the maximum figure on the 2nd day. Urea rose proportionately with the total nitrogen and ammonia was decidedly increased. Total phosphates were almost doubled. White cells dropped from about 14,000 to 2,000 after the injection. The bull-terrier did not seem to bear the injections so well as the preceding animal for on the 3rd day following this treatment there was vomiting, diarrhea, and refusal of food. Determinations were omitted during this period. This dog also tended to lose weight throughout the experiment. 1 kilo was lost during this period so that creatinine which was high in the control period was considerably lower when the second injection of 17 millicuries was given.

Probably because of the decided effect produced by the first treatment 3 weeks previously, this small dose caused an appreciable rise in total nitrogen with a proportional increase in urea, ammonia, and creatinine, which was evident for 3 days. Total phosphates were somewhat increased.

10 days after the second treatment 54 millicuries were injected. This dose produced no general effect except that the animal refused part of the food on the 4th day. Total nitrogen, urea, and ammonia were considerably increased for 3 days and remained above normal for 10 days. Creatinine was not affected.

TABLE II.

Period.	Date.	Weight of dog.	Urine.										Titratable 0.1 N acid.	Temperature.	Remarks.
			Volume.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Creatinine N.		P_2O_5			
	1919	kg.	cc.		gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	cc.	°F.	
Control.	June 4	12.6	168	1.028	3.73	3.22	86.18	0.132	3.53	0.177	4.73	0.624	60	101.5	
	" 5		158	1.032	3.86	3.31	85.86	0.115	2.98	0.171	4.43	0.624	44	101.0	Red cells 6,400,000 White " 13,800 Hemoglobin 85%
	" 6		288	1.018	3.78	3.28	86.52	0.179	4.74	0.177	4.67	0.707	64	101.0	Red cells 5,924,000 White " 14,400
	" 7		270	1.021	3.90	3.42	87.62	0.202	4.14	0.181	4.64	0.851	84	100.8	Hemoglobin 85%
I*	" 8–9		610	1.019	3.60	3.08	85.47	0.165	4.61	0.177	4.91	0.852	81	101.7	
	" 10	12.5	300	1.020	4.34	3.68	84.69	0.141	3.23	0.181	4.17	0.790	60	101.3	Red cells 5,600,000 White " 8,900
	" 11		280	1.025	4.52	3.79	83.85	0.224	4.96	0.175	3.87	1.31	96	100.6	Hemoglobin 85% Red cells 5,280,000 White " 4,550
	" 24		250	1.018	3.96	3.39	85.67	0.146	3.68	0.150	3.80	0.540	52	99.7	Hemoglobin 80%
	" 25		180	1.022	3.76	3.24	86.18	0.157	4.17	0.158	4.21	0.416	40		
	" 26		188	1.023	4.01	3.43	86.66	0.134	3.35	0.167	4.16	0.416	44	100.6	
	" 27		250	1.020	4.21	3.70	87.77	0.137	3.26	0.163	3.88	0.457	44	100.1	
	" 28	11.4	382	1.015	4.10	3.48	85.00	0.151	3.68	0.163	3.90	0.436	32	100.4	
II†	" 29–30		538	1.020	4.04	3.44	85.22	0.166	4.37	0.161	4.00	0.540	44	100.6	Red cells 4,976,000 White " 5,700
	July 1		243	1.022	4.56	3.86	84.55	0.213	4.67	0.179	3.92	0.623	72	100.4	Hemoglobin 85% Red cells 5,250,000
	" 2		248	1.020	4.22	3.60	85.21	0.204	4.85	0.177	4.19	0.603	56	102.5 100.6	White " 4,450 Hemoglobin 85%

	July 3		250	1.020	4.46	3.87	86.18	0.182	4.07	0.171	3.83	0.561	60	100.2	
	" 4	11.9	140	1.030	4.10	3.62	88.45	0.165	4.04	0.156	3.82	0.540	48		
	" 5		150	1.025	4.14	3.64	88.50	0.174	4.20	0.158	3.82	0.499	64		
	" 6–7		400	1.022	4.11	3.60	87.62	0.148	3.61	0.149	3.62	0.561	52	100.3	
	" 8		220	1.020	4.13	3.64	88.17	0.151	3.67	0.152	3.68	0.540	56		
III‡	" 9	11.9	155	1.028	4.14	3.63	87.62	0.162	3.93	0.158	3.82	0.519	56		
	" 10		360	1.020	4.83	4.27	88.37	0.154	3.19	0.163	3.38	0.665	56	101.4	Red cells 5,800,000 White " 3,650 Hemoglobin 85%
	" 11		390	1.017	4.80	4.18	87.08	0.199	4.15	0.158	3.29	0.665	68	101.7	
	" 12	11.4	220	1.026	4.87	4.20	86.22	0.235	4.84	0.158	3.25	0.644	60		
	" 13		205	1.025	4.43	3.92	88.43	0.148	3.35	0.162	3.64	0.561	52		
	" 14		295	1.017	4.15	3.54	85.21	0.207	5.00	0.163	3.93	0.520	52	100.9	
	" 15		325	1.017	4.50	3.81	84.54	0.223	5.17	0.162	3.59	0.561	52		
	" 16		385	1.016	4.43	3.77	84.90	0.219	4.94	0.156	3.53	0.541	60		
	" 17	11.6	300	1.018	4.48	3.90	87.10	0.231	5.16	0.163	3.64	0.492	45	100.9	
	" 18		335	1.017	4.55	3.95	86.78	0.193	4.26	0.153	3.37	0.478	56		
	" 19		328	1.017	4.66	3.97	85.16	0.185	3.98	0.155	3.32	0.499	52	101.3	
	" 20–21		550	1.018	4.13	3.52	85.37	0.168	4.08	0.152	3.68	0.478	40	100.8	Red cells 5,814,000 White " 4,100 Hemoglobin 80%
	" 22		415	1.014	4.24	3.57	84.28	0.190	4.50	0.153	3.62	0.519	64		
	" 23	11.4	348	1.017	4.42	3.76	85.10	0.224	5.08	0.152	3.43	0.561	68		
IV§	" 24		410	1.013	4.17	3.67	88.03	0.176	4.24	0.158	3.79	0.478	40	102.7	
	" 25‖		575	1.017	4.88	4.19	85.89	0.146	2.98	0.139	2.85	1.08	60	101.1	Red cells 5,200,000 White " 2,600 Hemoglobin 85%

* Injected 120 millicuries of active deposit at 7.15 p.m. on June 9.
† " 17.3 " " " " 2.45 " " " 30.
‡ " 54.7 " " " " 4.45 "
§ " 146.4 " " " " 3.00 "
‖ No food eaten after this date.

2 weeks later, the last injection of 146 millicuries was given. All food was refused for 4 days, and when on the 5th day more food was eaten severe diarrhea immediately followed. The dog lost 1.5 kilos during this time. She was then anesthetized and an autopsy made.

DISCUSSION AND SUMMARY.

Our results record the effect of a purely physical agent, radium emanation, upon the metabolism. The experiment with Dog 1 is the most satisfactory, since in this animal the dosage employed was not great enough to produce general indisposition or sickness.

In every instance in both experiments the injection of active deposit was followed by an increased output of nitrogen, reaching the maximum figure on the 2nd day after the treatment. Urea fluctuates with the total nitrogen, but the absolute as well as relative amount of the total nitrogen excreted as ammonia nitrogen is decidedly increased, especially on the 2nd day after the treatment.

Much interest may be attached to the marked increase in creatinine which is noted in Dog 1. The quantity of this constituent is usually independent of volume and of total nitrogen. Shaffer (3) found some time ago that increased temperature had a tendency to increase creatinine but that it afterward went below normal. Van Hoogenhuyze and Verploegh (4) found an increase of 50 per cent in creatinine with no rise in total nitrogen in cases with a temperature. Leathes (5) found, on the other hand, that creatinine increased 20 per cent, but the total nitrogen increased 50 per cent in fevers. He concluded that the proportion of nitrogen excreted during fever appearing as uric acid was considerably increased but that as creatinine was consistently diminished. In our case the creatinine elimination does not fluctuate with temperature change—in fact the first increase in creatinine is noted after the second injection when the temperature has returned to normal. Uric acid at this time showed no increase. Although the highest creatinine values are noted on the days after the treatment when the nitrogen also is high, they do not increase proportionally to the total nitrogen. On the 2nd day of Period II nitrogen increased 3.5 per cent and creatinine 14 per

cent, and in Period III nitrogen increased 17 per cent while creatinine increased 28 per cent. On this day creatinine was 45 per cent higher than in the control period at the beginning of the experiment. The proportion of nitrogen as creatinine nitrogen was somewhat increased, but not greatly, after the treatment.

Uric acid showed a marked increase both absolutely and relatively following injection of radioactive deposit.

The effect of the injection seems to be cumulative because the third treatment in Dog 1, only a little larger than the second injection, produced a very marked effect, while the fourth treatment, although smaller than the first, produced nausea.

The writers wish to express their appreciation to the Radium Department for supplying the radium emanation and to Dr. Stanley R. Benedict for his suggestions during the work.

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THE DETERMINATION OF THE REFRACTIVITY OF HEMOGLOBIN IN SOLUTION.

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INTRODUCTION.

The refractivity of proteins in solution has been extensively investigated by Reiss, Herlitzka, Schmidt, and by Robertson and his collaborators. An excellent summary and discussion of these results, together with a bibliography, have been published by Robertson.[1] The following summary statement of facts recapitulates the main aspects of this work as far as they bear on the present problem.

(*a*) Proteins dissolved in water increase the refractive index of the solvent by an amount nearly proportional to the concentration of the protein. This approximately linear relation is expressed by the formula[2] $n - n_1 = a \times c$, where n represents the refractive index of the solution, n_1 the refractive index of the solvent, a a constant characteristic of the protein, and c the concentration of the protein. To determine the value of a for a given protein the refractive index of an aqueous solution of that protein in known concentration is first determined; from this value is subtracted the refractive index of the solvent, and the remainder is divided by the concentration; the quotient resulting is the value of a. If the value of a is known the percentage of that protein in a solution of unknown concentration can be calculated from the same formula.

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[1] Robertson, T. B., The physical chemistry of the proteins, New York, 1918, 359.

[2] This formula does not express with entire precision the relation between n and c. It is based on the law of Gladstone and Dale which itself is only an approximation to experimental facts. It does, however, give the relation between n and c with a degree of inaccuracy not greater than the inherent error of refractometry under the conditions of this investigation.

(*b*) It has also been shown that the increase in refractive index produced by a protein is not sensibly affected by the presence of other substances in solution provided no considerable molecular change in the protein, or of its degree of hydration or dispersion, is produced. Therefore the concentration of a protein in solution with other proteins, or with acids, bases, or salts in low concentration, can be determined by observing the index of refraction of the solution with and without the protein in question.

(*c*) The value of a for a considerable number of proteins has been determined. These values range from 0.0023 to 0.0013.

(*d*) There are certain limitations to accuracy in refractometric readings. The Pulfrich refractometer has an inherent error of 0.00009, while the error of the Abbe instrument is about the same, being approximately 0.0001. It is obvious therefore, as Robertson remarks, that, since the absolute error in the determination of $n - n_1$ is the same, the error in the determination of a must be less in proportion to the magnitude of c. It is therefore desirable to use as concentrated solutions as possible when determining the value of a. But maximum concentrations of proteins cannot be used because when a certain concentration is exceeded the border line seen in the field of the instrument becomes hazy and accurate setting of the instrument is impossible.

The present investigation was undertaken to find out whether the concentration of hemoglobin in solution might be estimated by the refractometric method, and to establish the value of a for hemoglobin. If this could be done it was obvious that a useful method might be devised for estimating hemoglobin in blood.

Methods.

The index of refraction was measured by an Abbe refractometer by Zeiss loaned by the Physics Department of Williams College through the courtesy of Prof. W. E. McElfresh. This type of refractometer, while less commonly used than is that of Pulfrich, is nevertheless very satisfactory. If allowed to attain the temperature of the room in which it is used, and if the room temperature is kept approximately constant, there is no difficulty in making several successive observations at the same temperature. The prisms are, however, water-jacketed and can be connected with a constant temperature reservoir if desired. This was not found necessary in the present work. All readings were made at 19–21°C., and in determining the value of $n - n_1$ the two readings necessary were made in quick succession and therefore at the same temperature. As the apparatus is provided with a compensating device to correct dispersion, a sodium flame or other source of monochromatic light is unnecessary. In this work an ordinary incandescent lamp was used as the source of light. The scale of the instrument is so constructed that the index of refraction

can be read directly from the graduated arc. The smallest subdivisions of the scale represent intervals of 0.001, but with some practice it is possible to estimate tenths of a subdivision with fair accuracy. It has therefore been assumed in this work that the error in reading is $\pm$ 0.0001, but with care it is probable that the error can be reduced below this value. Several settings and readings were made for each determination and the average was taken. Consequently figures extending to the fifth decimal have occasionally been used with some confidence.

The work undertaken can be conveniently described in four parts; *viz.*, (*A*) preparation of pure hemoglobin, (*B*) examination of the validity of the formula $n - n_1 = a \times c$ when applied to hemoglobin solutions, (*C*) investigation of the possible effect of dilute alkalies and of serum constituents on the refractivity of hemoglobin, and (*D*) determination of the value of a for hemoglobin.

(*A*) *Methods of Preparation of Hemoglobin.*

Most of the crystallized hemoglobin used in this investigation was made by the method of Hoppe-Seyler as modified by Hüfner,[3] and especially as modified in some details by Butterfield.[4] In the case of hemoglobin from the guinea pig and the rat crystallization from laked blood took place readily in the cold without the addition of alcohol. Preparations from these species were, however, of little value for the purpose in hand because of their very sparing solubility. In the case of dog, horse, and beef bloods, alcohol was added to the laked blood, after cooling to 0°C., to make a concentration of from 20 to 30 per cent. The actual effective concentration was uncertain, and may have been higher than 30 per cent in some cases as ice crystals usually separated out at the low temperatures employed ($-$ 10 to $-$ 20°C.) thus raising the concentration of alcohol in the solution. Crystallized hemoglobin was separated by centrifugalization, washed twice with 25 per cent alcohol, and finally dried on a porous plate *in vacuo* over H_2SO_4 at or close to 0°C.

Hemoglobin prepared in this way always contained a small and variable proportion of material insoluble in water but soluble in dilute alkali. This probably consisted of serum globulin and of stromata of corpuscles that had escaped solution by the ether, and that had been carried down by hemoglobin crystals in the centrifuge. It was found that after apparently thorough hemolysis of corpuscles by ether it was still possible to demonstrate stromata in the apparently clear liquid. If a drop of this liquid was mixed with a drop of saturated ammonium sulfate solution and examined under the microscope it was always possible to find some stro-

[3] Hüfner, G., *Arch. Physiol.*, 1894, 134.

[4] Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173.

mata, often in the typical bell-shaped form of erythrocytes. The salt did, of course, precipitate some of the hemoglobin, but there was no difficulty in distinguishing the stromata from the granular hemoglobin precipitate.

An effort was made to remove the stromata by filtration, but without success. Shaking the laked blood with filter paper pulp or with siliceous earth followed by filtration through asbestos felt at four atmospheres pressure was ineffective. As long as the solution came through the filter at all it carried stromata with it, but the flow stopped after a few cc. had filtered through. Centrifugalization was wholly ineffective in separating the stromata, as other investigators also have remarked.

When, however, hemoglobin prepared as above described was dissolved in water it was possible to separate the insoluble impurity by centrifugalization, to wash, dry, and weigh it, and then to calculate its percentage in the hemoglobin. But such a procedure probably gives inaccurate results and cannot be adopted with satisfaction.

After numerous unsuccessful efforts to get entirely consistent results with hemoglobin made as described, the paper of Welker and Williamson[5] appeared calling attention to the method of removing colloids from solution devised by Marshall and Welker.[6] The latter authors had shown that it is possible to remove entirely colloids *other than hemoglobin* from solutions by treatment with aluminum hydroxide. With this method, it was found that by shaking a laked corpuscle preparation with a freshly prepared mass of aluminum hydroxide, the colloids, other than hemoglobin, were adsorbed and suspended matter was entangled by the hydroxide and that a perfectly clear solution of hemoglobin, together with salts, could be filtered off with the utmost readiness. This method is of such excellence as to warrant the opinion that much of the work on hemoglobin in the past, in which discordant results have been obtained, might well be repeated using the method of Marshall and Welker for the preparation of the pure substance. All the observations included in the results published in the present paper, except where otherwise stated, were made on hemoglobin prepared from solutions thus purified. The technique of preparation was essentially that of Butterfield with the addition of the aluminum hydroxide purification after laking the blood and before the addition of alcohol.

[5] Welker, W. H., and Williamson, C. S., *J. Biol. Chem.*, 1920, xli, 75.

[6] Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820.

(*B*) *Examination of the Validity of the Formula* $n - n_1 = a \times c$ *for Hemoglobin.*

As has already been stated, the refractivity of most proteins in solution is a linear function of their concentration. When the optical behavior of hemoglobin in this respect was examined it was found that within the limit of the inherent experimental error, the refractivity was directly proportional to the concentration. Experiment 1 demonstrates this agreement with the law.

Experiment 1.—The material used for this experiment consisted of twice washed horse corpuscles, laked with ether, then freed from ether by aspirated air, and finally treated with aluminum hydroxide. It contained about 17 per cent of hemoglobin and a small amount, probably about 1 per cent, of salts. 1 cc. of this solution was placed in each of six tubes, and to each tube was added from a calibrated pipette of 3 mm. bore enough distilled water to make the dilution 90, 80, 70, 60, 40, and 20 per cent respectively in the several tubes. After the liquid in each tube had been mixed, the refractive index was measured as well as the refractive index of the original solution. Table I gives the results of this experiment.

TABLE I.

	$n - n_1$	$\frac{n - n_1}{\text{relative concentration}}$
Original solution	0.0310	0.0031 ± 0.00001
90 per cent dilution	0.0282	0.0031 ± 0.00001
80 " " "	0.0251	0.0031 ± 0.00001
70 " " "	0.0216	0.0031 ± 0.00001
60 " " "	0.0187	0.0031 ± 0.00002
40 " " "	0.0124	0.0031 ± 0.00003
20 " " "	0.0062	0.0031 ± 0.00005

(*C*) *Refractivity of Hemoglobin in Water, in Dilute Alkali, and in Serum.*

As it was desired to use as solvents both dilute alkali and serum, it was necessary to find out whether the refractivity of hemoglobin was the same in each of the solvents. It had been shown by Gladstone and Dale[7] that in general the refractivity of

[7] Gladstone, J. H., and Dale, T. P., *Phil. Trans. Roy. Soc.*, 1858, cxlviii, 8; 1863, cliii, 316.

a solute is uninfluenced by other solutes provided no molecular change is induced, and Robertson[8] has shown that this general truth applies to protein. The latter has further shown that the relatively slight alteration of molecular structure that occurs when proteins unite with acids or bases does not sensibly alter the refractivity, but, on the other hand, that the change in the degree of hydration that occurs when amorphous serum albumin solution is half saturated with ammonium sulfate does cause a notable change in the refractive index. With these facts in mind there would seem to be no reason to anticipate that the refractivity of hemoglobin in dilute alkali or in serum would be different from that in water, but the result of the experiment is conclusive. The refractivity of hemoglobin in these solvents is normal.

Experiment 2.—The refractive index of a solution of hemoglobin of about 17 per cent strength, and that of a 0.1 N NH_4OH solution were first determined, 1 cc. of each of these solutions was mixed together, and the refractive index of the mixture determined. It is evident that if the refractivity of the hemoglobin remained normal in the presence of 0.05 N NH_4OH then the refractive index of the mixture would be equal to the mean of the refractive indices of the component solutions. A parallel experiment with hemoglobin and serum was also made. The results follow.

Hemoglobin in Dilute Alkali.

(1) n of hemoglobin solution	1.3661
(2) n of 0.1 N NH_4OH	1.3341
Mean of refractive indices of (1) and (2)	1.3501
n of mixture of equal parts of (1) and (2)	1.3501

Hemoglobin in Serum.

(1) n of hemoglobin solution	1.3667
(2) n of serum	1.3440
Mean of refractive indices of (1) and (2)	1.35535
n of mixture of equal parts of (1) and (2)	1.3553

(*D*) *Determination of the Value of a for Hemoglobin.*

1. By Means of Solutions of Pure Crystallized Hemoglobin.—

Experiment 3.—Tubes 8 by 120 mm. each containing a glass bead were prepared and weighed. Crystallized dry hemoglobin was weighed in the

[8] Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179.

tubes and the tubes were nearly filled with 0.1 N NH_4OH. The amount of hemoglobin was chosen to make approximately a 10 per cent solution. Solution was effected by warming in a bath at 30°C. and by thorough shaking. The tube was then centrifugalized to destroy the foam and a few drops of the solvent were added to facilitate observation of the meniscus. The level of the meniscus was carefully marked on the tube, the con-

TABLE II.

Concentration of Hb.	$n - n_1$	a
9.66	0.0178 ± 0.0001	0.001842 ± 0.00001
10.89	0.0198 ± 0.0001	0.001819 ± 0.00001
Average		0.001830 ± 0.00001

TABLE III.

Source of blood.	Concentration.	$n - n_1$
Guinea pig.	2.08	0.00365
	2.45	0.00425
Rat.	2.00	0.00320
	4.30	0.00770
Dog.	3.63	0.00630
	3.75	0.00610
	3.93	0.00700
	4.30	0.00755
	9.30	0.01800
Horse.	6.19	0.01160
	7.66	0.01360
Ox.	2.77	0.00485
	2.95	0.00555
	6.26	0.01065
	7.06	0.01275
	7.89	0.01385

Average value of $a = 0.00178 \pm 0.00002$. In determining the value of a from a number of observations due allowance should be made for the greater accuracy of the values obtained from the more concentrated solutions; *i.e.*, a weighted average should be calculated. Following Robertson's suggestion this has been done by dividing the sum of the values of $n - n_1$ by the sum of the values of c.

tents were thoroughly mixed, and the refractive index was read. The tube was then washed and filled with distilled water to the mark, and weighed. The volume of the tube to the mark divided into the weight of hemoglobin gave the concentration of hemoglobin in the solution of which the refractive index had been read. From these data the value of a was calculated. Table II gives the results of two determinations on horse hemoglobin.

Before becoming acquainted with the aluminum hydroxide method of preparing pure hemoglobin, sixteen separate determinations of the value of a were made on specimens of hemoglobin prepared from the blood of the rat, guinea pig, dog, horse, and ox. It seems worth while to include the results of these observations (see Table III), although the value of a obtained is affected by the presence of the insoluble impurity to which reference has already been made, and is consequently slightly lower than the correct value given in Table II.

2. By Means of Hemoglobin Solutions Freed from Salts by Dialysis but Not Crystallized.—The hemoglobin molecule is very sensitive to denaturing influences. While the uniformity of results obtained by different recent observers who have determined the spectrophotometric constant of hemoglobin would incline one to the conclusion that they were dealing with a sufficiently stable substance, yet the divergence of results of proximate analyses make it possible that either the alcohol used to bring about crystallization, or the subsequent drying, results in a preparation that is to some extent dehydrated or denatured, and possibly with an altered refractivity. With this possibility in view the value of a was calculated from hemoglobin that had not been subjected to any dehydrating agent and that had not been crystallized. The following procedure was employed.

Experiment 4.—Ether-laked blood was purified by aluminum hydrate and then dialyzed in a Schleich and Schüll thimble until the dialysate gave no precipitate with $AgNO_3$, and its refractive index was the same as that of pure water. Such a solution is free from colloids and suspended matter, and sufficiently free from salts for the purposes of this investigation. The value of $n - n_1$ (n_1 of distilled water) for this solution was determined and then an accurately measured volume was evaporated in a drying oven, and the weight of the dry residue determined in the usual way. The concentration of hemoglobin was calculated from the volume of the solution and the weight of dry residue. The following result was obtained.

Concentration of Hb. *per cent*	$n - n_1$	a
13.23	0.0242	0.00183 ± 0.000008

3. By Refractometric Comparison of Laked and Unlaked Blood, with Gasometric Determination of Hemoglobin.—Unlaked blood offers an exceptionally favorable opportunity for measuring the refractivity of hemoglobin; for in unlaked blood the hemoglobin, remaining in the corpuscles, does not affect the refractive index, the refractive index of whole blood being identical with that of the plasma.[9] On laking, the hemoglobin is discharged from the corpuscles, and entering into solution in the plasma raises its refractive index by an amount proportional to the percentage of the hemoglobin in the blood. This statement involves the assumption that freezing and thawing cause, first, the complete discharge of all the hemoglobin, and, second, an inappreciable discharge of electrolytes and water. The first assumption can be readily tested. It was found that alternately freezing and thawing blood three times caused a rise in the refractive index that was not increased by further freezing and thawing, and from blood so treated no sediment could be obtained on centrifugalization. The extent to which water and electrolytes are discharged from the corpuscles by hemolysis cannot be so easily determined. Stewart[10] has observed the change in conductivity of serum following hemolysis produced by various agencies, and has concluded that freezing and thawing cause a less discharge of electrolytes than does any other agency. He was unable to estimate with precision the possible increase of electrolytes in the serum because the entrance of hemoglobin itself into the serum lowers the conductivity by opposing the migration of ions and by putting back the dissociation of electrolytes. From the results of Stewart's work, however, it seems probable that any change in the refractivity of the serum due to the entrance of water and electrolytes from the corpuscles must be of a very low order of magnitude. Moreover the concordance between the result of the present experiment, based on this assumption,

[9] When whole blood is first observed in the refractometer the border line is very hazy, but within 30 seconds it becomes quite sharply defined.

[10] Stewart, G. N., *J. Physiol.*, 1899, xxiv, 211.

and the results of other methods of determining the value of a speaks for the correctness of the assumption itself.

The gasometric method of determining the oxygen of blood devised by Van Slyke[11] furnishes an accurate method of estimating the percentage of hemoglobin, for it is known that 1 cc. of oxygen combines with 0.744 gm. of hemoglobin.[12]

Experiment 5.—The oxygen capacity of human blood, oxalated and thoroughly oxygenated by rolling for 20 minutes in a large bottle, was measured in the Van Slyke apparatus. Only two drops of octyl alcohol, redistilled at 35 mm. pressure and tested, were used, and saponin was dispensed with to make it practicable to use so small an amount of foam preventer. The average of five closely concordant readings was 0.418 cc. The calculated amount of oxygen was 16.97 cc. per 100 cc. of blood, and the calculated percentage of hemoglobin 12.625 (16.97×0.744).

1 cc. of the same blood was sealed in a tube and alternately frozen and thawed four times. The refractive index of the perfectly clear liquid was then measured and found to be 1.3697. The refractive index of the whole blood (or of the plasma) was 1.3466. The difference between these two figures, the refractivity of the hemoglobin, is 0.0231, and this number divided by the concentration of the hemoglobin (12.625) gives 0.00183 ± 0.00008, which is the value of a.

CONCLUSIONS.

The object of this investigation was the determination of the value of a for hemoglobin. When reached by three separate and distinct methods the result has proved to be the same; namely, $a = 0.00183$. It is therefore believed that this figure can safely

[11] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347; 1918, xxxiii, 127.

[12] It has been conclusively shown by Peters (Peters, R. A., *J. Physiol.*, 1912, xliv, 131) and by Barcroft and Burn (Barcroft, J., and Burn, J. H., *J. Physiol.*, 1913, xlv, 493) that loosely combined oxygen and iron are associated in oxyhemoglobin in the proportion of 1 molecule of oxygen to 1 atom of iron; and Butterfield (Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173) has determined the percentage of iron in oxyhemoglobin to be 0.335 per cent. Calculation from these data shows that 1 cc. of oxygen is combined with 0.744 gm. of hemoglobin.

No account has been taken in this work of the theoretical difference in the refractivities of oxyhemoglobin and hemoglobin. As molecular refractivity is an additive function of atomic refractivity the introduction of 2 atoms of oxygen into the enormous hemoglobin molecule would affect the refractivity to a quite inappreciable extent only.

be used to calculate the strength of hemoglobin solutions of unknown concentration when the value of $n - n_1$ can be found.

With the value of a determined, and with the knowledge that the difference in the refractive indices of laked and unlaked blood is due practically entirely to the hemoglobin, it is evident that a simple and fairly accurate clinical method of estimating hemoglobin in blood can be devised. The details of a clinical method, employing saponin hemolysis, are now being worked out. This technique together with data regarding the degree of accuracy obtainable will be communicated later.

SUMMARY.

1. It has been shown that the refractive index of solutions of hemoglobin varies directly with the concentration.

2. The refractive index of hemoglobin in aqueous solution has been shown to be independent of the presence of other proteins, and of bases and salts in low concentration.

3. The value of a for hemoglobin has been determined to be 0.00183.

4. The possibility of a practical clinical method for the refractometric estimation of hemoglobin has been indicated.

The advice and collaboration of Professor G. A. Shook, now of Wheaton College, Norton, Mass., but at Williams College at the beginning of this investigation in 1917, were of great value. On the resumption of the investigation, interrupted by the war, Professor Shook's absence made his cooperation no longer possible. He has, however, independently devised and reported[13] a differential refractometer which it is believed can be conveniently used with this method.

The facilities of the Chemical Laboratory of Williams College were made available when necessary for this work through the kindness of Professor Brainerd Mears.

[13] Shook, G. A., *J. Ind. and Eng. Chem.*, 1918, x, 553.

NUTRITIVE FACTORS IN PLANT TISSUES.*

IV. FAT-SOLUBLE VITAMINE.

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

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(Received for publication, February 16, 1920.)

The occurrence of fat-soluble vitamine in green foods has been demonstrated for alfalfa, clover, and timothy plants and for the leaves of spinach and cabbage.[1] From some of these we have obtained potent products by drying the plant tissues in a current of air at about 60° and then extracting them with U. S. P. ether.[2] The resultant green extracts from spinach leaves, young clover, alfalfa, and grass respectively yielded an oily residue approximating 3 per cent of the dried plant.[3] These residues, fed in daily quantities equivalent to 1 to 2 gm. of the dried plant, promoted recovery and renewal of growth in rats declining in weight on diets deficient in fat-soluble vitamine. McCollum, Simmonds, and Pitz[4] have stated that "ether extraction of plant tissue does not remove the substances essential for growth which is contained in butter fat." The results of our successful experience,

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] For a discussion of the literature see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187.

[2] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 98.

[3] The actual content of substance removed from a number of samples of dried plant tissues in this way was as follows: spinach, 3.0 and 4.3 per cent; young alfalfa, 3.0 per cent; green grass, 4.1 per cent; timothy, 4.2 per cent; tomato, 2.8 per cent.

[4] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 363.

however, are illustrated in Chart I. The food mixtures had the composition as given in Table I.

Although dried tomato was efficacious as a source of fat-soluble vitamine, its ether extract was not (see Chart II).

Recently Steenbock and Gross[5] have concluded that:

". . . . tubers and roots are not necessarily to be classed with food materials grossly deficient in their fat-soluble vitamine content. While in some instances it is true that there is little or no fat-soluble vitamine demonstrable, in other instances there is enough present to warrant their classification with respect to their content of this dietary essential with leafy materials rather than with our cereal grains such as maize, wheat, barley, or oats."

TABLE I.

Food.	Rats 5411, 5412, 5417, 5425, 5430.	Rats 5570, 5563.	Rat 5527
	per cent	*per cent*	*per cent*
Meat residue	19.6		
Casein		18.0	
Lactalbumin			18.0
Salt mixture*	4.0	4.0	4.0
Starch	52.4	48.0	47.0
Lard	24.0	30.0	31.0
Yeast	0.2–0.6 gm. daily.	0.4 gm. daily.	0.4 gm. daily.

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

We are not familiar with any published determinations of the *absolute* quantity of carriers of fat-soluble vitamine necessary to keep growing animals in health and vigor. It has been reported that for rats 2 per cent of butter fat is sufficient for the maintenance of good growth when all other dietary factors are of good quality,[6] although Drummond[7] who has had large experience in this field has failed to secure normal growth of rats with even 4 per cent of butter fat in an otherwise suitable diet. Early in our studies of the fat-soluble vitamine we[8] secured good growth for

[5] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 529.

[6] McCollum, E. V., Simmonds, N., and Parsons, H., *J. Biol. Chem.*, 1919, xxxvii, 162.

[7] Drummond, J. C., *Biochem. J.*, 1919, xiii, 81, Experiments 1 to 10, 85.

[8] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

long periods on diets containing 1 and 3 per cent of butter fat respectively along with protein-free milk; ultimately a decline ensued and body weight was restored by increasing the content of butter fat in the food mixture.

Without knowledge as to the total food intake it is impossible to estimate the absolute amount of butter fat eaten with any approach to accuracy. However, rats usually eat enough of diets thus poor in fats to make it unlikely that the minimum quantity of butter fat ingested daily in the case of the 2 per cent fat ration would be less than 150 to 200 mg.

In contrast with this we have observed that only 42 mg. per day of the U. S. P. ether extract of grass sufficed to furnish enough fat-soluble vitamine to promote renewal of growth in rats that had declined on diets deficient in this factor. Furthermore in a number of instances the now familiar eye disease that frequently afflicts rats which have been for some time on a diet deficient in fat-soluble vitamine was cured after the administration of ether extracts of alfalfa, grass, or spinach (see Chart I, Rats 5417, 5570, 5425, 5563).

The foregoing observations have raised the question as to the comparative potency of green vegetables, roots, *etc.* as sources of fat-soluble vitamine in contrast with the more familiar carriers represented in the fats of milk, egg, *etc.*—products already demonstrated to furnish this essential food factor. To secure evidence we have fed rats, beginning with approximately the same age and size, on a diet complete in respect to each known essential except fat-soluble vitamine. The basal food mixture consisted of:

	per cent
Meat residue	19.6
Salt mixture*	4.0
Starch	52.4
Lard	24.0
Yeast	0.4 gm. daily.

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The quantity of brewery yeast added as a source of water-soluble vitamine has been demonstrated by us to be ample and

also practically free from fat-soluble vitamine.[9] Upon the diet outlined rats soon fail to grow and then begin to decline rapidly in body weight, as shown in Chart III, Rats 5496, 5491, and 5512. Unless the physiological damage has proceeded too far, restoration of growth and well being is usually readily brought about by inclusion of butter fat in place of part of the lard in the food mixture. The characteristic eye conditions or symptoms, sometimes termed xerophthalmia or perhaps better keratomalacia, which often develop during the decline in health,[10] are speedily relieved when the nutritive conditions begin to improve.

To test the comparative efficacy of various products as sources of fat-soluble vitamine they were fed, apart from the food mixture, in daily doses of approximately 0.1 gm. of the dried substance.[11] These were consumed with readiness by the animals so that a fairly constant supply of the product to be tested was always ingested, despite variations in the total food intake.

The products used were prepared from the plant materials by heating in a large drier, through which a current of air circulated at 60° or less, and grinding the dry residues to a powder.

The changes in body weight of the animals in this series of experiments are shown in Charts IV to XI. Whenever any of the animals began to decline in body weight and thus give evidence of unsatisfactory nutrition the diet was changed by the addition of 18 per cent of butter fat to the food mixture, in order to ascertain whether a shortage of the fat-soluble factor was the real cause for the failure of maintenance.

To permit comparison with a more familiar source of fat-soluble vitamine a series of rats was fed 0.1 gm. of butter fat daily instead of the dried vegetable. With a food intake of 50

[9] We have referred to this fact in earlier papers; *c.g.*, Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 158; 1919, xxxvii, 199. See also Drummond, J. C., *Biochem. J.*, 1917, xi, 255.

[10] We cannot accept the implication of Bulley, E. C., *Biochem. J.*, 1919, xiii, 103, that lack of fat-soluble vitamine plays no decisive rôle in the appearance of the symptoms of eye disease. Our evidence will be presented at another time.

[11] The materials were measured with small scoops which did not deliver exact quantities. The ± variations were found subsequently to amount to 40 per cent in a few instances. This does not materially alter the significance of the results.

to 70 gm. per week, which we[12] have found usual for animals of the same size on adequate foods of similar calorie value, this amount of butter fat would be equivalent to that in a mixture containing 1.4 to 1.0 per cent thereof. Chart III contrasts the records of rats that received no butter fat and began to decline in 50 to 60 days with the weight curves of three rats which were fed 0.1 gm. of butter fat daily and reached a maximum weight of 290, 310, and 320 gm. respectively before giving any indication of qualitative inadequacy of their diet. Rat 5300 could no longer be restored by the administration of butter fat; the other two animals, however, showed a slight response to this product, indicating that they may have suffered somewhat from a lack of the fat-soluble factor found therein.

The growth of rats to adult size upon diets in which the daily intake of butter fat as a source of fat-soluble vitamine was as small as in the experiments just described was surprisingly good. It should be borne in mind that, in contrast with what is true of animals having declined through complete deprivation of some requisite food factor, the problem of dosage for vitamines may be quite different in the case of rats which are supplied with a minimum of the essentials throughout the period of growth. When the other dietary factors were satisfactory we have repeatedly found 0.5 gm. of butter fat per day to be sufficient to restore to good nutritive condition and growth rats declining on diets devoid of fat-soluble vitamine. This corresponds, for our food mixtures, to a butter fat content of about 5 per cent. How much less might suffice, we cannot state at present.

If butter fat, which has been studied more extensively than any other source of fat-soluble vitamine, is used as a standard for comparison the relatively large content of fat-soluble vitamine in some of the vegetable products examined is at once suggested by inspection of our records. For example, two rats (Nos. 5385 and 5386, Chart IV) receiving only 0.1 gm. of tomato daily have rapidly grown to exceptionally large adult size and have not shown any signs of a failure of nutrition at the end of 394 days. In considering this unexpectedly good physiological performance it must be remembered that the more rapid growth

[12] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

at the start, may have been facilitated by the additional supply of water-soluble vitamine (water-soluble B) furnished by the tomato, which we[13] have demonstrated to be rich in this factor.

It may be necessary to take into consideration another factor supplied by the dried tomato. Givens and McClugage[14] have shown that the latter, in contrast with some other vegetable foods, may retain a significant amount of its antiscorbutic potency. Is the exceptionally good growth of the tomato-fed rats attributable to this? Although it has generally been assumed that rats are not susceptible to scurvy both Harden and Zilva[15] and Drummond[16] maintain that this species also requires antiscorbutic vitamines. Thus Harden and Zilva conclude:

". . . . rats existing on a scorbutic diet, although capable of gaining in weight and reproducing themselves, without any apparent manifestation of pathological symptoms for months, do not thrive so well as animals which have their diets supplemented with an antiscorbutic. This suggests that although rats are not very susceptible to scurvy they cannot absolutely dispense with antiscorbutics without restriction of their normal development."

The evidence of all these English investigators consists in the demonstration of the attainment of larger size by rats which received orange juice or lemon juice as an antiscorbutic in addition to yeast as a source of water-soluble vitamine. It is not clear, however, that the fruit products did not function merely as added sources of water-soluble B which we have found them to contain,[17] and thus promote the rate of growth by the increment of a factor other than the antiscorbutic. We have frequently observed improved growth in rats when the supply of vitamine from yeast was increased. Without a demonstration that more yeast, which is not regarded as antiscorbutic, or some other source of water-soluble B should fail to accomplish what is claimed for orange juice added to yeast, the conclusion of the

[13] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; 1920, xli, 451.

[14] Givens, M. H., and McClugage, H. B., *J. Biol. Chem.*, 1919, xxxvii, 253.

[15] Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 408.

[16] Drummond, J. C., *Biochem. J.*, 1919, xiii, 77.

[17] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 46.

need of some special antiscorbutic by rats is not convincing. We have on several occasions attempted without success by giving orange juice or tomato to promote the growth of rats that failed to respond properly to yeast as a source of water-soluble vitamine. However, it should be pointed out that occasionally animals which fail to grow on our food mixtures as well as one might expect begin to thrive when a mixed diet is furnished. The reason for this is not yet clear.

Among the various dried vegetables tested for their comparative content of fat-soluble vitamine the cabbage was least satisfactory (see Rats 5247, 5248, 5297, Chart V). The ready response of two of the animals to additions of butter fat to the diet indicates the character of the deficiency in the cabbage. 0.1 gm. of alfalfa, clover, timothy, and spinach evidently furnishes relatively at least as much of this vitamine as does 0.1 gm. of butter fat (see Charts VI, VII, VIII, and IX). These vegetable products may in fact contain more than butter fat.

Some of the animals receiving the dried green products grew to large adult size before evincing any signs of a possible shortage of fat-soluble vitamine. Eye disease characteristic of a lack of this vitamine was not observed in any of these animals.

Tests with dried carrots are shown in Chart X. The experiments with potato (including the skin), Chart XI, are not strictly comparable with the others inasmuch as the quantities of the tuber used, *i.e.* 20 per cent of the food, were large. The large size attained by two of the rats on the potato food indicates that the tuber as a whole cannot be entirely devoid of fat-soluble vitamine.

In an investigation of the distribution of fat-soluble vitamine in some roots Steenbock and Gross[5] have recently concluded:

> "With 15 per cent of the diet made up of roots as the source of the fat-soluble vitamine we have in the case of the yellow sweet potato and carrot normal growth and even rearing of the young made possible, but in the case of the rutabaga, dasheen, red beet, parsnip, potato, mangel, and sugar beet complete failure resulted."

In the experiments of these investigators larger quantities of potato in the diet, however, permitted growth, this corresponding with our observation that this tuber, though poor in fat-soluble vitamine, is by no means entirely devoid of it.

Early in our investigation of the properties of butter fat we reached the conclusion that the fat-soluble vitamine as it occurs in this natural product is not readily destroyed by heating with steam. "Butter fat through which live steam was passed for two and one-half hours or longer did not lose its characteristic restorative properties"[18] when fed to rats which had declined on diets deficient in fat-soluble vitamine. We have since duplicated this observation.

Steenbock, Boutwell, and Kent[19] and Drummond[7] have reached the conclusion that the fat-soluble vitamine is readily destroyed by heat. Thus Drummond states, in confirmation of the claims of the American investigators:

"Exposure of butter fat to 100° for periods of from one to four hours destroys its growth promoting power entirely, so far as can be determined by experiments on young rats . . . . The effect of lower temperatures was also investigated and it was ascertained that the nutritive value of butter fat may be appreciably lowered by four hours' exposure to temperatures ranging from 50°–75°."[20]

In comparing these statements with the findings which we published earlier it should be noted that the newer experiments have involved heating the fat *in the absence of water*. Our heated butter fat was subjected to steaming, not dry heat, for several hours. It seemed possible that the differences in the mode of heating, involving unlike possibilities of dehydration, *etc.*, might account for the discrepancies recorded. However, in experiments which will be reported in another paper we have since heated dry butter fat in an air bath at 96° for 15 hours without destroying sufficient fat-soluble vitamine (if any) to make the product appear inferior to the original butter fat when tested on rats that have declined from lack of fat-soluble vitamine.

We are not prepared to say that heat is without effect upon this vitamine, because we have not yet tested heated butter fat in quantities sufficiently small to meet the valid objection that despite some destruction sufficient amounts still remained in the

[18] Osborne and Mendel,[8] p. 381.

[19] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

[20] Drummond,[7] p. 86.

large portion of heated fat fed to satisfy all requirements. We do know, however, that our animals *ate* all the heated butter fat apportioned to them, because it was fed apart from the food and not incorporated in the rest of the ration. In considering Drummond's experiments one is struck by the fact that even with 6 per cent of unheated butter fat in the diet[21] his control rats grew at much less than the normal rate. This is contrary to the experience of several investigators, including ourselves, and raises a question as to the value of the untreated butter fat or the food intake of the animals used by him. At any rate the reason for the discrepancies between us are not apparent.

We have referred here in detail to some of these problems in connection with the effect of heat on the fat-soluble vitamine because it might be assumed that the low heat employed in desiccating the fresh foods studied in this research had diminished their content of fat-soluble vitamine. It is known that cabbage loses its antiscorbutic potency through drying; and this naturally suggests that the poor showing made by dried cabbage as a source of fat-soluble vitamine might also be ascribed to deterioration through heat and desiccation. The positive results secured with the other dried foods speak against such a conclusion. We have not yet made the crucial comparisons between the fresh and dried products. Drummond[7] states in respect to his own observations that "the experiments with cabbage gave evidence that drying may reduce the efficiency of leaves as a source of fat-soluble A, but no definite opinion can yet be given." Drummond refers to the experiments on guinea pigs by Delf[22] and Delf and Skelton[23] which lead them to believe that high temperature or the drying of cabbage leaves may effect a destruction of the fat-soluble accessory. Their experiments, however, seem to us to lack adequate controls and are not convincing. More recently Steenbock and Gross have asserted, in contrast with their findings regarding the thermolability of the vitamine in butter fat, that "the fat-soluble vitamine as found in plant materials was very stable to heat."[24]

[21] Drummond,[7] p. 86, Experiment 6, Table I.

[22] Delf, E. M., *Biochem. J.*, 1918, xii, 416.

[23] Delf, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 448.

[24] Steenbock and Gross,[5] p. 506.

The newer studies indicating the richness of many types of plant tissues in those nutritive properties termed vitamines place the dietary importance of the green vegetables in an entirely new light. It emphasizes their use to supplement the refined foods of the modern food industry which furnish products rich in proteins, fats, and carbohydrates but in many cases comparatively deficient in the vitamines. The facts cited in the present investigation, along with others recently published, serve as an added reminder that the fat-soluble vitamine need not be sought solely in foods known to be rich in fats.

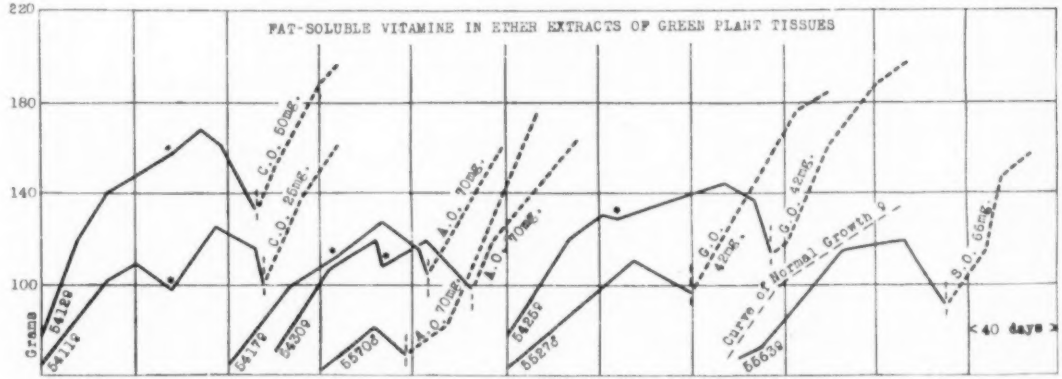

CHART I. Showing the efficiency of U. S. P. ether extracts from clover, alfalfa, grass, and spinach respectively as sources of fat-soluble vitamine in promoting restoration of growth in rats that had begun to decline on diets otherwise adequate. An asterisk (*) marks the period at which the daily allowance of dried brewery yeast, fed apart from the rest of the food, was increased to 0.6 gm. Note that this addendum alone sufficed to promote the growth of the animals for a time in some instances, although decline ultimately ensued in every case. Rats 5417, 5570, 5425, and 5563 developed the characteristic eye disease, which in each case disappeared after the administration of the plant oil. Interrupted lines indicate the periods during which the plant oils were fed mixed with starch and apart from the rest of the ration. C.O. = clover oil; A.O. = alfalfa oil; G.O. = grass oil; S.O. = spinach oil. The daily dose is indicated on the chart.

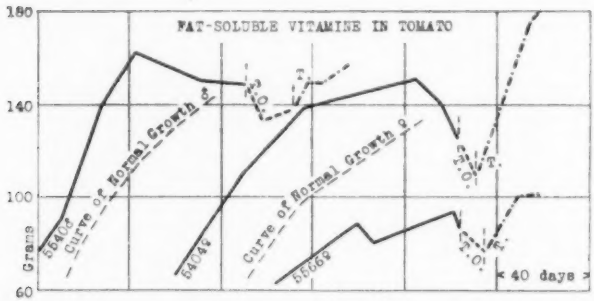

CHART II. Showing the failure of the ether extract of dried tomato (T. O.), in the quantities used, to promote renewal of growth in animals that had declined on a diet deficient in fat-soluble vitamine; and the renewal of growth when 1 gm. of dried tomato (T.) was furnished daily. The composition of the foods was as follows:

	Rat 5440♂.	Rat 5404♀.	Rat 5566♀.
	per cent	*per cent*	*per cent*
Casein.....	18.0		18.0
Meat residue....		19.6	
Salt mixture*....	4.0	4.0	4.0
Starch.....	48.0	52.4	48.0
Lard.....	30.0	24.0	30.0
Yeast.....	0.4 gm. daily.	0.2–0.6 gm. daily.	0.4 gm. daily.
Tomato oil.....	56–112 mg. "	56 mg. "	112 mg. "

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

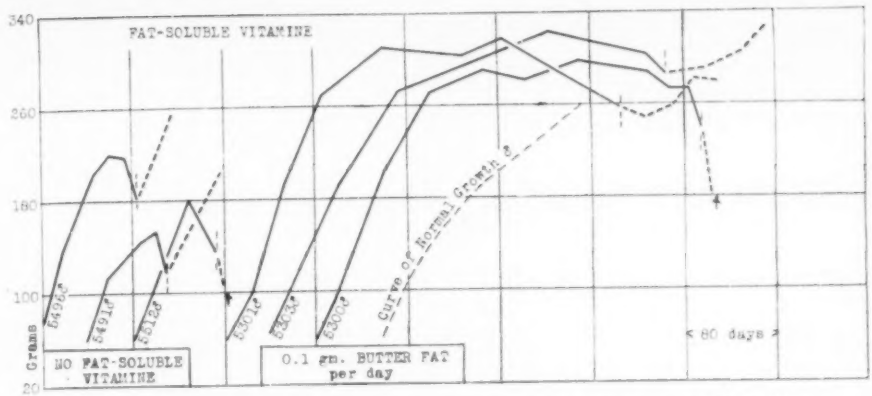

CHART III. Showing early failure to grow and decline of Rats 5496, 5491, and 5512 on a diet deficient in fat-soluble vitamine; also better growth of Rats 5301, 5303, and 5300 when 0.1 gm. of butter fat was supplied daily from the beginning of the experiment. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

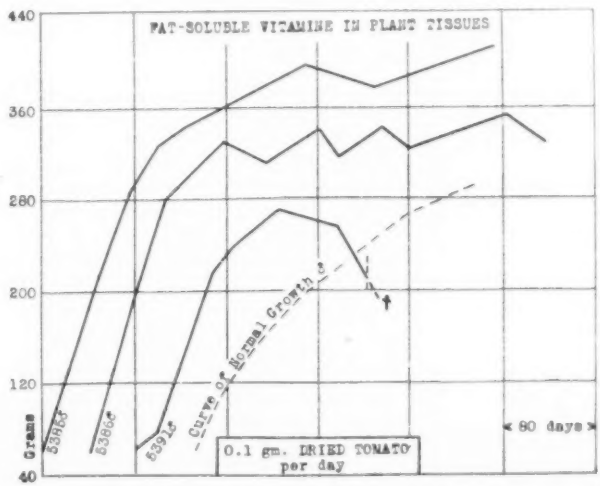

CHART IV. Showing the exceptional growth of rats on a diet containing 0.1 gm. of dried tomato as the source of fat-soluble vitamine. They represent the most successful growth observed in the entire series in this paper. Rat 5391 died of lung disease.

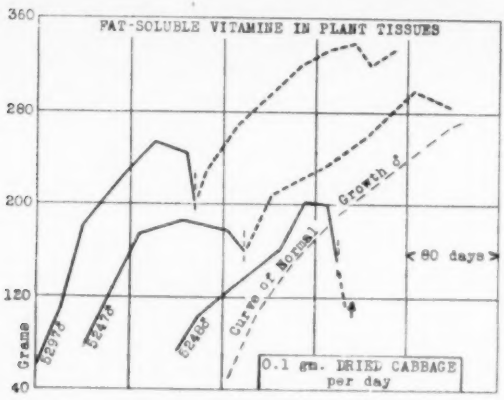

CHART V. Showing the inadequacy of 0.1 gm. of dried cabbage per day to supply sufficient fat-soluble vitamine to permit prolonged growth. The nature of the deficiency is shown by the renewal of growth when 18 per cent of butter fat was included in the diet during the period represented by the interrupted lines. That this amount of cabbage is not entirely lacking in fat-soluble vitamine is shown by the contrast of these results with the control experiments without fat-soluble vitamine, represented in Chart III.

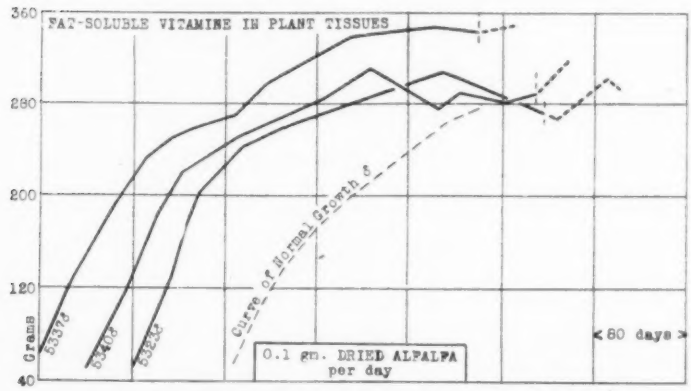

CHART VI. Experiments in which 0.1 gm. of dried alfalfa was fed daily in addition to a diet deficient in fat-soluble vitamine. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

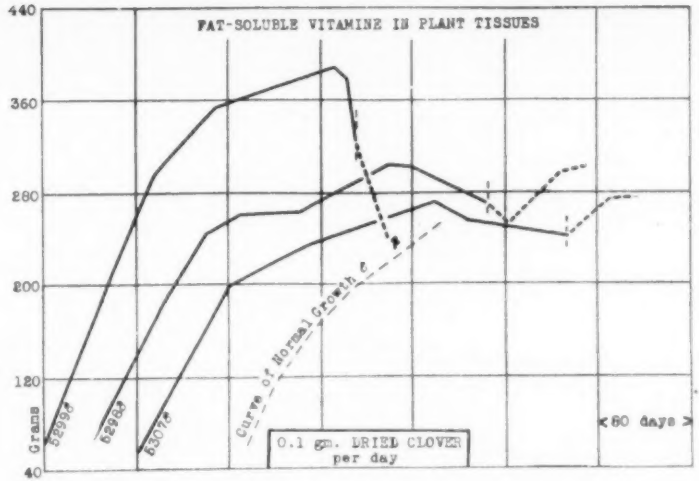

CHART VII. Experiments in which 0.1 gm. of dried clover was fed daily in addition to a diet deficient in fat-soluble vitamine. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet. Rat 5299 died of lung disease.

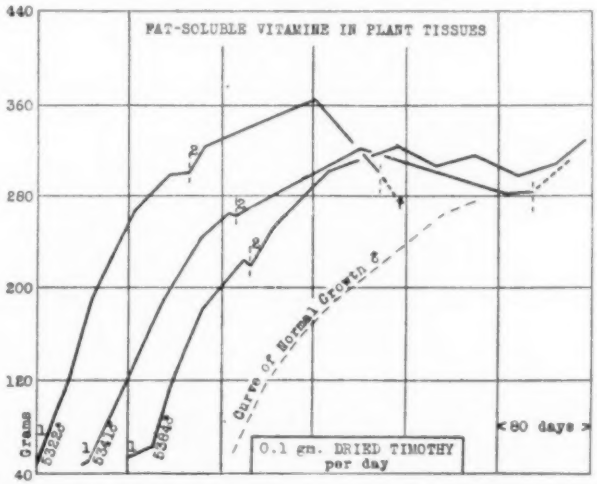

CHART VIII. Experiments in which 0.1 gm. of dried timothy (during Period 1) or 0.1 gm. of dried mixed grasses (during Period 2) was fed daily in addition to a diet deficient in fat-soluble vitamine. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

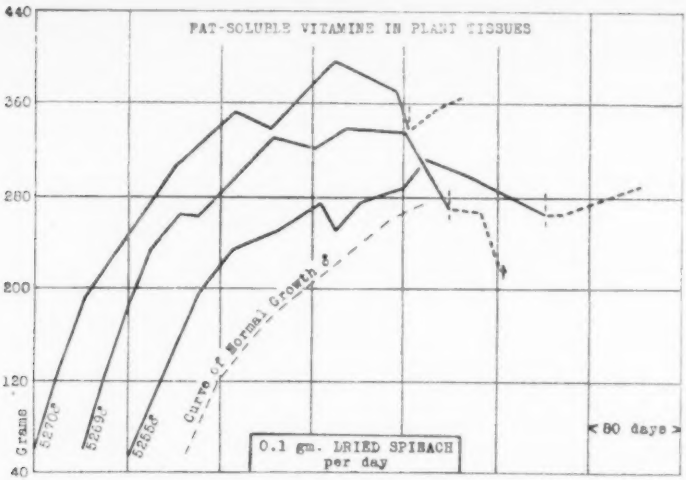

CHART IX. Experiments in which 0.1 gm. of dried spinach was fed daily in addition to a diet deficient in fat-soluble vitamine. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet. Rat 5269 died of lung disease.

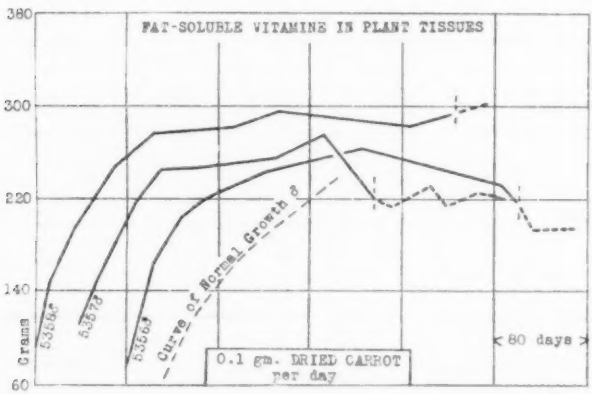

CHART X. Experiments in which 0.1 gm. of dried carrot was fed daily in addition to a diet deficient in fat-soluble vitamine. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

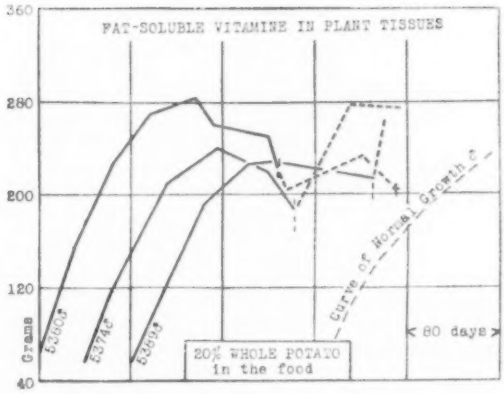

CHART XI. In these experiments the food consisted of

	per cent
Meat residue	15
Whole potato	20
Salt mixture*	4
Starch	36
Lard	25
Yeast	0.2 gm. daily

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The fat-soluble vitamine was furnished by the dried potato until the period represented by the interrupted line, during which 0.5 gm. of butter fat per day was fed in addition. Rat 5380 died of lung disease.

STUDIES OF ACIDOSIS.

XVI. THE TITRATION OF ORGANIC ACIDS IN URINE.

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Description of Method.

100 cc. of urine, roughly measured, are thoroughly mixed with 2 gm. of finely powdered calcium hydroxide, allowed to stand about 15 minutes with occasional stirring, and then passed through a dry folded filter. This treatment removes carbonates and phosphates. To 25 cc. of the filtrate in a 125 to 150 cc. test-tube of clear glass[1] one adds 0.5 cc. of 1 per cent phenolphthalein solution, and 0.2 N hydrochloric acid from a burette (amount need not be measured) until the pink color just disappears (pH = approximately 8). 5 cc. of 0.02 per cent tropeolin OO solution are then added. As the indicator solution is added it is thoroughly mixed with the urine by shaking the tube; if this precaution is omitted some of the tropeolin OO may be precipitated. Finally 0.2 N hydrochloric acid is added from the burette until the red color equals that of a standard solution containing 0.6 cc. of 0.2 N HCl, 5 cc. of tropeolin OO solution, and water to a total volume of 60 cc. When the end-point is approached, sufficient water is added to the titrated solution to make its volume equal to that of the 60 cc. standard solution used in a similar tube as a color control.

In comparing the color of the titrated solution with that in the standard, it is convenient during the titration to hold the two

[1] We use the tubes of Pyrex glass made by the manufacturers for urea determinations by the Van Slyke and Cullen technique. The tubes are 30 mm. inner diameter, 200 mm. long, and uniform in size.

tubes side by side between the thumb and fingers, the tube containing the urine being the one held nearer to the tips of the fingers where it can be easily shaken as the 0.2 N acid is run in from the burette.

Sometimes it is desirable to use a similar technique for the phenolphthalein end-point also. In this case a tube of urine filtrate to which no phenolphthalein is added serves as a standard.

We have found that as the final end-point with tropeolin OO is approached comparison of colors is somewhat facilitated by placing the two tubes side by side in a comparator of the form described by Dernby and Avery, although with practice the end-point may be located within 0.1 cc. by merely holding the tubes together as described above.

Calculation.

From the volume of 0.2 N HCl used to titrate from the end-point of phenolphthalein to that of the tropeolin OO, the amount, usually 0.7 cc., is subtracted which is utilized in a similar titration of a control determination in which water is substituted for the urine. The volume of 0.2 N HCl thus corrected represents the approximate organic acid content of the urine sample, plus the creatine and creatinine, and an amount of amino-acids ordinarily negligible.

In order to calculate the results in terms of cc. of 0.1 N organic acid per liter, the figure representing the cc. of 0.2 N HCl used in the titration is multiplied by 80 (by $\frac{1,000}{25} = 40$ in order to transfer figure from 25 cc. to 1,000 cc. of urine, and by 2 to change from 0.2 N to 0.1 N terms).

Correction for Creatinine.—A 0.1 M solution of creatinine (11.32 mg. per cc.) titrates in the above determination as a 0.1 N solution of organic acid. Therefore, in order to correct for the creatinine, the cc. of 0.1 N organic acid per liter calculated from the above titration may be diminished by

$$\frac{\text{mg. creatinine per liter urine}}{11.32} \text{ or by } \frac{\text{mg. creatinine N per liter urine}}{4.2}.$$

The simplest way is to subtract the creatinine correction directly from the cc. of 0.2 N acid used in the titration, and mul-

tiply the difference by 80. In this case the correction is $\frac{1}{80}$ as great as the above; *i.e.*, cc. correction = $\frac{\text{mg. creatinine per liter urine}}{906}$ or $\frac{\text{mg. creatinine N per liter urine}}{336}$.

Example.—

		cc.
0.2 N HCl used in titration		7.6
Correction found in blank analysis	0.7 cc.	
Creatinine correction for 500 mg. creatinine N per liter urine. Correction $= \frac{509}{336}$ cc. $=$	1.2 "	
Total correction	1.9 cc.	
Corrected titration figure $= 7.6 - 1.9$		5.7
0.1 N organic acid per liter $= 80 \times 5.7$		456.0

Tropeolin OO was preferred by us as indicator for the final end-point. In neutral solution it gives nearly the same yellow color as urine, but so much more intense that a water solution of 0.002 N hydrochloric acid with the indicator can be used as a color standard without the use of a comparator. Very dark urines may need greater dilution, but such are not often encountered. Another advantage of this indicator is that its maximum acid color is not reached even at pH 2.7, so that if too much HCl is added in the titration the solution becomes redder than the standard. This particular advantage is possessed in much less degree by the three indicators mentioned below as alternatives.

Other indicators that may be used are methyl orange, tetrabromophenolsulfonephthalein (bromophenol blue, Clark and Lubs), and dimethylaminoazobenzene. To some eyes the color change of one of these dyes may be more readily detected than that of tropeolin OO. The two azo dyes are not much different in color from tropeolin OO, both changing from yellow to red, but the bromophenol blue turns from blue to a clear yellow on acidifying, and affords a very different alternative. To the authors the tropeolin OO end-point appeared the most satisfactory, however.

Theoretical Basis of Method.

The method is based on the following previously known facts:

1. Relatively little strong mineral acid is required to change the hydrogen ion concentration of a water solution from 10^{-8} to 2×10^{-3} if the only electrolytes present are alkali salts of strong acids, such as sulfates and chlorides.

2. If the salt of a weak acid is present, however, the addition of nearly a full molecule of hydrochloric acid for each molecule of such salt is necessary in order to cause the above change in hydrogen ion concentration. The organic acids known to occur in normal and pathological urines, in amounts sufficient to be quantitatively significant in the total acid excretion of the body, belong to the class of weak acids whose salts behave in the above manner.

3. The only mineral acids found in significant amounts in urine which belong to the class of weak acids, and therefore form salts which show the above behavior, are phosphoric and carbonic acids.

4. Very weak bases form salts which behave like those of the weak acids. Creatinine is titrated almost quantitatively in changing the hydrogen ion concentration from 10^{-3} to 2×10^{-3}, and creatine to about 60 per cent. Aside from the traces of amino-acids, these appear to be the only bases of this kind present in considerable amount in human urine.

Effect of the Different Organic Acids of the Urine on the Titration.

The titration figure obtainable by titrating between two hydrogen ion concentrations a solution containing the salt of an acid of a known dissociation constant may be calculated as follows:

From the law of mass action:

$$(1)\quad H^+ = k\frac{HA}{A'}$$

H^+ = hydrogen ion concentration in terms of normality.
A' = anion of acid.
k = dissociation constant of the acid.
HA = free, undissociated acid.
BA = salt of the acid.
λ = degree of dissociation of the salt into Na and Ac.

When the salt of the acid is present, and dilutions are of the magnitudes used in titrations (0.1 to 0.01 M), the equation becomes practically

$$(2) \qquad H^+ = \frac{k}{\lambda} \times \frac{HA}{BA}$$

As λ in the high dilutions encountered approaches unity, it may in approximate calculations be neglected.

Equation 1 may then be expressed as

$$(3) \qquad H^+ = k \times \frac{HA}{BA} \text{ or } \frac{HA}{BA} = \frac{H^+}{k}$$

For acetic acid $k = 1.8 \times 10^{-5}$.

When pH equals 8, or $H^+ = 1 \times 10^{-8}$, we therefore have in the case of acetic acid $\frac{HC_2H_3O_2}{BC_2H_3O_2} = \frac{10^{-8}}{1.8 \times 10^{-5}} = \frac{1}{1{,}800}$. One part in 1,801 parts, or 0.05 per cent, of the acid is free.

When pH = 2.7, $H^+ = 2 \times 10^{-3}$, and we have $\frac{HC_2H_3O_2}{BC_2H_3O_2} = \frac{2 \times 10^{-3}}{1.8 \times 10^{-5}} = \frac{200}{1.8}$.

At a pH of 2.7, therefore, $\frac{200}{201.8}$, or 99.2 per cent, of the acid is free. Changing the hydrogen ion concentration of an acetate solution from the slightly alkaline reaction of 10^{-8} N (or a pH of 8) to the acid reaction of 2×10^{-3} ((pH = 2.7) approximately the reaction of 0.002 N HCl) therefore requires an amount of HCl equal in molecular equivalents to 99.15 per cent of the total acetate present.

For the different acids which occur or may occur in human urine, the values in Table I are calculated. The values of the constants are for 25° unless otherwise indicated.

Comparison of the results so calculated with those experimentally obtained in titrating solutions of some of these acids is satisfactory, as shown in Tables III, IV, and VI of the experimental part of this paper. The conclusion seems justified that the titration as carried out estimates certainly over 90 per cent of the organic acids of the urine, and presumably over 95 per cent, since

a higher titration value by 3 or 4 per cent is obtained for those acids excreted as ammonium salts. The data for carbonic and phosphoric acids indicate the necessity for their removal before the organic acids are titrated.

TABLE I.

Calculated Titration Values of Weak Acids of the Urine.

Acids.	Dissociation constant.	Acid free at		Calculated proportion of acid determined by titrating from pH 8 to pH 2.7.
		$H^+ = 10^{-8}$ N pH = 8	$H^+ = 2 \times 10^{-3}$ pH = 2.7	
		per cent	*per cent*	*per cent*
Organic.				
Uric*	1.5×10^{-6}	0.5	99.9	99.4
Acetic†	1.8×10^{-5}	0.0	99.2	99.2
β-hydroxybutyric‡	2.0×10^{-5}	0.0	99.0	99.0
Lactic†	1.4×10^{-4}	0.0	93.5	93.5
Acetoacetic‡	1.5×10^{-4}	0.0	93.1	93.1
Citric§	2.0×10^{-4}	0.0	91.0	91.0
Formic*	2.1×10^{-4}	0.0	90.6	90.6
Hippuric†	2.2×10^{-4}	0.0	90.2	90.2
Mineral.				
$H(NaHPO_4)$‖	2.0×10^{-7}	2.5	100	97.5
$H(HCO_3)$¶	3.5×10^{-7}	4.2	100	95.8

* His and Paul.

† Ostwald.

‡ Henderson and Spiro.

§ Shown by Amberg and McClure to occur in amounts equivalent to 60 to 70 cc. of 0.1 N acid in a normal 24 hour urine. The titration values for citric acid given in Column 5 are those directly determined by Sörensen. The constant is estimated from them.

‖ Sörensen.

¶ Kendall.

Effect of Weak Bases of the Urine on the Titration.

The amount of strong acid required to change the pH of a solution of a weak base from 8 to 2.7 may be calculated from the dissociation constant K_b.

$$K_b = OH' \times \frac{\text{salt of base}}{\text{free base}} = \frac{10^{-14}}{H^+} \times \frac{\text{salt of base}}{\text{free base}}$$

The "salt of base" represents the amount combined with acid. At pH 8, therefore, salt of base, or $\frac{\text{acid combined with base}}{\text{free base}} = K_b \times \frac{10^{-8}}{10^{-14}} = K_b \times 10^6$. At pH 2.7, or $H^+ = 2 \times 10^{-3}$, the ratio is $K_b \times \frac{2 \times 10^{-3}}{10^{-14}} = 2\ K_b \times 10^{11}$. The difference between the acid bound by a given base at pH 8 and that bound at pH 2.7 represents the amount required to titrate between the two points. Table II contains a list of the weak bases of the urine, with their constants and the proportion of an equivalent of HCl

TABLE II.

Calculated Titration Values of Organic Bases of the Urine.

Base.	Basic dissociation constant. K_b	Base free at		Proportion estimated by titrating with HCl from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-3}$	
		$H^+ = 10^{-8}$	$H^+ = 2 \times 10^{-3}$	Calculated.	Observed.
		per cent	*per cent*	*per cent*	*per cent*
Urea*.....	0.0015×10^{-11}	100	99.7	0.3	0.2
Creatinine†..	1.81×10^{-11}*	100	24.0	76.0	99
Creatine‡....	3.57×10^{-11}*	100	12.3	87.7	60
Ammonia‡....	1.5×10^{-5}†	6.2	0.0	6.2	5.3-6.0

* Walker and Wood.

† Measured at 40°, Wood.

‡ Noyes, Kato, and Sosman.

required to titrate each from pH 8 to pH 2.7, calculated as above indicated. The constants are from data obtained at 25°, except for creatine and creatinine. In Column 5 results are brought forward from Table V, showing the amounts of HCl bound by the different bases in the titration, as determined experimentally.

Urea is, both by observation and calculation, practically without effect on the results of the titration, even when the urea concentration is at the maximum observed in human urine.

The available data on the K_b of creatine and creatinine do not yield calculated results corresponding so closely with those experimentally obtained as do the data on the other substances requir-

ing consideration. The divergence is perhaps due to the fact that Wood's values for K_b of creatine and creatinine were determined at 40°, while the titration is performed at 20°. It is evident, however, that practically all the creatinine is titrated as organic acid. The amount of this substance excreted varies between 13 and 27 mg. per kilo of body weight per 24 hours (Folin, 1905). The mean, 20 mg., would neutralize 1.8 cc. of 0.1 N acid per kilo or 108 cc. for a 60 kilo individual.

Creatine when present titrates to about 60 per cent as an organic acid; but it is excreted by adults only in conditions involving rapid autolysis of muscle tissue, and would therefore not, as a rule, require consideration.

Ammonia is titrated to the extent of 5 to 6 per cent, but the actual effect of the presence of organic acids as ammonium rather than fixed alkali salts is to make the results of the titration with most of the acids approximate more closely the theoretical values, as shown in Table IV. The ammonium salts of the organic acids titrate 2.3 to 4.6 per cent more completely than the sodium salts, not 6.2 per cent more completely, as would be theoretically expected, and as is approximately realized for the ammonium salts of hydrochloric and sulfuric acids. The observed positive ammonia error is such as to make the results obtained with all but the weakest organic acids approximate more closely to 100 per cent than the results obtained in the absence of ammonia. The tendency of the ammonia error to correct the opposite error in the organic acid titration is enhanced by the fact that ammonia and organic acid excretion tend to run parallel, particularly when acid excretion is abnormally high, as in diabetic acidosis. For the reasons, therefore, that the ammonia correction is not great and is of a nature actually to diminish, as a rule, the other error in the determination, it has seemed not only simpler but better to attempt no correction for it in urine analyses.

Effect of Amino-Acids on the Titration.

Amino-acids if present in large amount would be disturbing factors, as at an H^+ of 2×10^{-3} they bind with their NH_2 groups considerable amounts of acid. Glycocoll, which does not differ much from the other monoamino-acids in this respect, binds

about $\frac{1}{3}$ molecule of HCl at this H^+. The amount is calculated as follows:

The acid constant for glycocoll is 3.4×10^{-10}, the basic constant 2.9×10^{-12}, as calculated by Winkelblech from conductivity measurements. From the acid constant we have by calculating as above:

— COOH free at $H^+ = 10^{-8}$ N	— COOH free at $H^+ = 2 \times 10^{-3}$ N	Proportion of COOH group estimated by titration from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-3}$
per cent	*per cent*	*per cent*
96.7	100	3.3

The function of the NH_2 group is similarly calculated from the basic constant, $K_b = 2.9 \times 10^{-12} = (OH)' \times \frac{\text{glycine chloride}}{\text{free glycine}}$ or

$$\frac{COOH\text{-}CH_2\text{-}NH_2}{COOH\text{-}CH_2\text{-}NH_2\,HCl} = \frac{(OH)'}{2.9 \times 10^{-12}} = \frac{10^{-14}}{H^+ \times 2.9 \times 10^{-12}} = \frac{10^{-2}}{H^+ \times 2.9}.$$

From these values we calculate:

— NH_2 free at $H^+ = 10^{-8}$ N	— NH_2 free at $H^+ = 2 \times 10^{-3}$ N	Proportion of NH_2 group estimated by titratin with HCl from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-3}$
per cent	*per cent*	*per cent*
100	63.3	36.7

The total consumption of HCl by both COOH and NH_2 groups in the titration should be, according to the above calculation, $0.03 + 0.367 = 0.40$ molecule of HCl per 1 molecule of glycine present. The actual amount observed by Sörensen was 0.385 molecule.

The other monoamino-acids apparently bind similar amounts of HCl. The constants for leucine and alanine were determined by Winkelblech as follows: leucine, $K_a = 3.1 \times 10^{-10}$, $K_b = 2.7 \times 10^{-12}$; alanine, $K_a = 9.0 \times 10^{-10}$, $K_b = 3.8 \times 10^{-12}$. According to these, leucine would require in the titration 0.38 molecule of HCl; alanine 0.36, nearly the same as glycocoll. The results in Table VII for the mixture of all the monoamino-acids obtained from casein are in the same neighborhood (44 per cent).

The amino-acid nitrogen constitutes 1 to 2 per cent of the total urinary nitrogen (Van Slyke, 1913–14; Henriques). On a daily excretion of 14 gm. of nitrogen, 2 per cent would indicate 200 cc. of 0.1 M amino-acids. The neutralizing power of such an amount of amino-acids in the titration would be about 80 cc. of 0.1 N hydrochloric acid.

Our knowledge of the nitrogenous constituents of the urine indicates the presence of no weak bases, aside from those discussed, in quantities sufficient to affect markedly the organic acid titration under discussion, and the nitrogenous excretory products have been so thoroughly studied that it is unlikely that any quantitatively important substances with definitely basic properties have been overlooked.

It therefore appears that in titrating the 24 hour urine of an adult of average size for organic acids, as described in this paper, about 100 cc. of the 0.1 N organic acid estimated is in reality due to creatinine and creatine, 80 cc. or less to amino-acids, and the remainder to organic acids.

EXPERIMENTAL.

Titration of Organic Acids in Water Solutions.—A 20 cc. portion of each acid, of approximately 0.1 N concentration, was titrated in a 100 cc. test-tube with either 0.1 N sodium hydroxide or 0.1 N ammonium hydroxide to neutrality with 0.5 cc. of 1 per cent phenolphthalein. 1 cc. of 0.1 per cent tropeolin OO was then added, and the solution titrated back with 0.2 N HCl to pH 2.7, using 0.002 N HCl solution as standard. The results are given in Tables III and IV.

Titration of Weak Bases in Water Solutions.—Solutions of the bases in 25 cc. portions were brought to pH 8 by addition of 0.1 N NaOH or 0.2 N HCl until a barely visible pink color was reached; then tropeolin OO was added and the solution titrated to pH 2.7. The results are given in Table V.

Effect of Concentration of Phenolphthalein on its End-Point in Presence of Ammonium Salts.—The concentration of phenolphthalein to some extent affects the pH at which the pink color is just visible. If there is but little indicator present a greater part of it must be in the colored form to give a perceptible pink

TABLE III.

Titration of Sodium Salts of Organic Acids.

Acid	(A) 0.1 N NaOH to neutralize acid to phenolphthalein.	(B) 0.2 N HCl to titrate back to pH 2.7 with tropeolin OO.	(C) Average 0.2 N HCl corrected for blank.	(D) Organic acid determined. $\frac{200\ (C)}{(A)}$	Organic acid theoretically titratable from pH 8 to pH 2.7 (from Table I).
	cc.	*cc.*	*cc.*	*per cent*	*per cent*
Blank	0.1	0.50	0.00		
Acetic	20.00	10.60	9.95	99.5	99.4
		10.50			
Citric	19.86	9.30	8.88	89.4	91.0
		9.35			
Lactic	20.28	9.90	9.40	92.7	93.5
		9.90			
Hydrochloric	20.00	0.70	0.20	1.0	

TABLE IV.

Titration of Ammonium Salts of Organic Acids.

Acid.	(A) 0.1 N acid present.	(B) 0.1 N NH_4OH to neutralize acid to phenolphthalein	0.2 N HCl to titrate back to pH 2.7.	(D) Average 0.2 N HCl corrected for 0.5 cc. blank.	(E) Proportion of organic acid determined $\frac{200\ (D)}{(A)}$	Proportion of NH_4 salt theoretically titratable; i.e., that for acid calculated in Table I + 6.2 per cent for NH_4 present.	Differences between average percentage of Na salt and NH_4 salt titrated.
	cc.	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*
Acetic	19.68	20.51	10.70	10.25	104.1	105.6	4.6
	19.68	20.47	10.80				
Citric	21.04	21.55	10.30	9.79	93.0	97.2	3.5
	21.04	21.51	10.27				
Lactic	20.06	20.96	10.03	9.50	94.7	99.7	2.3
	20.06	20.96	10.03				

than when the total amount of indicator is greater. Consequently the amount of extra alkali required to make a solution of an ammonium salt show pink with phenolphthalein is some-

TABLE V.

Observed Behavior of Weak Bases when Titrated from pH 8 to pH 2.7.

Base.	Amount present in the 25 cc. of solution titrated.		0.2 N HCl required in titrating from pH 8 to pH 7.	Proportion of base titrated.	Proportion of base calculated as titratable from dissociation constant (Table II).
	gm.	*cc. 0.2 N*	*cc.*	*per cent*	*per cent*
Urea	1.000	83.3	0.1	0.12	0.3
Creatine	0.200	7.6	4.1	60.0	87.7
Creatinine	0.100	4.41	4.32	97.8	76.0
	0.200	8.83	8.80	99.7	
Monoamino-acids	0.100	7.37*	3.25	44.2	36.0–40.0 for glycine, leucine, and alanine.
from casein	0.200	14.63	6.37	43.5	
	0.200	14.63	6.29	43.0	
Ammonia (as		12.50	0.67	5.4	6.2
$(NH_4)_2SO_4$)		12.50	0.75	6.0	
Ammonia (as		9.82	0.53	5.4	6.2
NH_4Cl)			0.52	5.3	

* Calculated on a nitrogen content of 10.3 per cent. The preparation was made by hydrolyzing casein with sulfuric acid, precipitating the bases with phosphotungstic acid, and concentrating the filtrate to dryness under reduced pressure after the phosphotungstic and sulfuric acids had been removed.

TABLE VI.

Effect of Phenolphthalein Concentration on End-Point in Presence of Ammonium Salts.

0.05 M $(NH_4)_2SO_4$.	1 per cent phenolphthalein.	0.1 N NaOH to turn pink to phenolphthalein.	0.2 N HCl to change from phenolphthalein end-point to pH 2.7.		Proportion of ammonia titrated from phenolphthalein end-point to pH 2.7
			Uncorrected.	Minus 0.5 cc. for correction.	
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*
25	0.1	0.85	1.42	0.92	7.3
25	0.2	0.65	1.36	0.86	6.9
25	0.5	0.45	1.20	0.70	5.6
25	1.0	0.45	Too cloudy with precipitated phenolphthalein to titrate.		

what dependent on the amount of indicator used. This is shown by the results in Table VI. It is desirable to use in performing the titrations 0.5 cc. of 1 per cent phenolphthalein solution, as directed, rather than the indefinitely measured drop or two which suffices in ordinary titrations.

TABLE VII.

Titration of Organic Acids Added to Urine.

Organic acid added.	0.1 N organic acid added to 100 cc. urine.	0.2 N HCl used in duplicate titrations of 25 cc. urine filtrate.	Average titration figure minus that for urine alone.	0.1 N added organic acid per liter diluted urine.		Proportion of added organic acid determined.
				Found.	Added.	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*
Acetic.	0	3.00 3.00				
	25	4.55 4.53	1.54	123	125	98.4
	50	6.20 6.15	3.17	253	250	101.2
	100	9.15 9.10	6.13	490	500	98.0
Lactic.	0	2.87 2.87				
	25	4.25 4.20	1.36	109	117*	93.2
	50	5.50 5.60	2.68	214	236*	90.7
	100	8.30 8.25	5.41	432	472*	91.6

* The 0.1 N lactic acid used in this experiment had the factor 0.945.

Titration of Known Amounts of Organic Acids Added to Urine.—100 cc. portions of a mixed sample of normal urine were mixed with portions of 25, 50, and 100 cc. respectively of acetic or lactic acid. Each mixture was then diluted to 200 cc., and 100 cc. portions were treated as previously described for determination.

TABLE VIII.

Excretion of Organic Acids with Creatinine Correction.

Data from hospital patients.

Subject.	Weight.	Condition.	Urine excretion.									
			Period	Volume.	Creatinine N.		0.2 N HCl used in titrating from pH 8 to pH 2.7.		0.1 N organic acid content.			
									Total.		Per kilo.	
							Duplicates.	Average minus 0.6 cc. correction for blank.	Uncorrected for creatinine.	Corrected for creatinine.	Uncorrected for creatinine.	Corrected for creatinine
	kg.		*hrs.*	*cc.*	*gm.*	*cc. 0.1 M*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Z	60	Myocarditis, decompensation on admission.	12 (day)	658	0.240	57	7.0, 6.9	6.35	334	277		
			12 (night)	946	0.261	62	4.3, 4.1	3.60	272	210		
			24	1,604	0.501	119			606	487	10.5	8.1
O	60	Myocarditis, some decompensation.	12 (day)	707	0.223	53	5.1, 5.25	4.57	258	205		
			12 (night)	744	0.216	51	5.0, 5.0	4.40	262	211		
			24	1,451	0.439	104			520	416	8.2	6.9
C	55	Chronic aortic endocarditis.	12 (day)	242	0.141	34	15.1, 15.3	14.60	282	248		
			12 (night)	332	0.195	46	12.8, 12.9	12.25	325	279		
			24	574	0.336	80			607	527	11.0	9.6
D	62	Chronic myocarditis with decompensation.	12 (day)	647	0.226	54	5.6, 5.7	5.05	261	207		
			12 (night)	750	0.196	47	4.4, 4.3	3.75	225	178		
			24	1,397	0.422	101			486	385	8.8	7.0
H	50	Orthostatic albuminuria.	12 (day)	364	0.091	22	5.55, 5.55	4.95	144	122		
			12 (night)	380	0.091	22	5.25, 5.10	4.48	136	114		
			24	744	0.182	44			280	236	5.6	4.7

The results are given in Table VII. The results are essentially the same as those obtained with acetic and lactic acids in pure water solutions.

Organic Acid Excretion by Individuals with Normal Metabolism.

The data given are sufficient only to indicate the usual excretion of organic acids; the possible normal variations, particularly under unusual conditions, may be greater. The figures of Table

TABLE IX.

24 Hour Excretion of Organic Acids by Normal Young Men.

Subject.	Weight.	24 hour urine.			
		Volume.	0.1 N organic acids uncorrected for creatinine.*		Total N.
	kg.	*cc.*	*cc.*	*cc. per kg.*	*gm.*
Ce	54.4	1,000	492	9.0	9.3
Dy	68.0	1,650	657	9.8	11.5
E	68.0	975	583	8.5	11.7
Fr	62.1	1,500	531	8.5	13.2
H	68.0	1,150	412	6.1	7.8
Sh	56.6	1,500	453	8.0	10.0
K	68.4	1,000	490	7.2	8.7
Sp	57.2	1,400	521	9.1	9.0
Fe	82.6	1,100	748	9.1	15.5
Dn	87.0	1,300	493	5.7	13.2
Ch	56.2	1,100	420	7.5	11.2
K	61.2	700	499	8.2	10.0
Ck	56.6	1,300	547	9.7	12.1
Average				8.2	

* The creatinine correction would reduce the total organic acid figure by about 2 cc. per kilo.

VIII are from afebrile heart patients, with apparently normal metabolism. The day periods are from 6 a.m. to 6 p.m., the night periods from 6 p.m. to 6 a.m. The data of Table IX are from a series of healthy young men. The figures indicate that the usual excretion of organic acids uncorrected for creatinine varies from about 280 to 750 cc. of 0.1 N acid per 24 hours, or from 5.6 to 11 cc. per kilo of body weight. The creatinine correction reduces the figures to 240 to 600 and 4.7 to 9.6 cc. respectively.

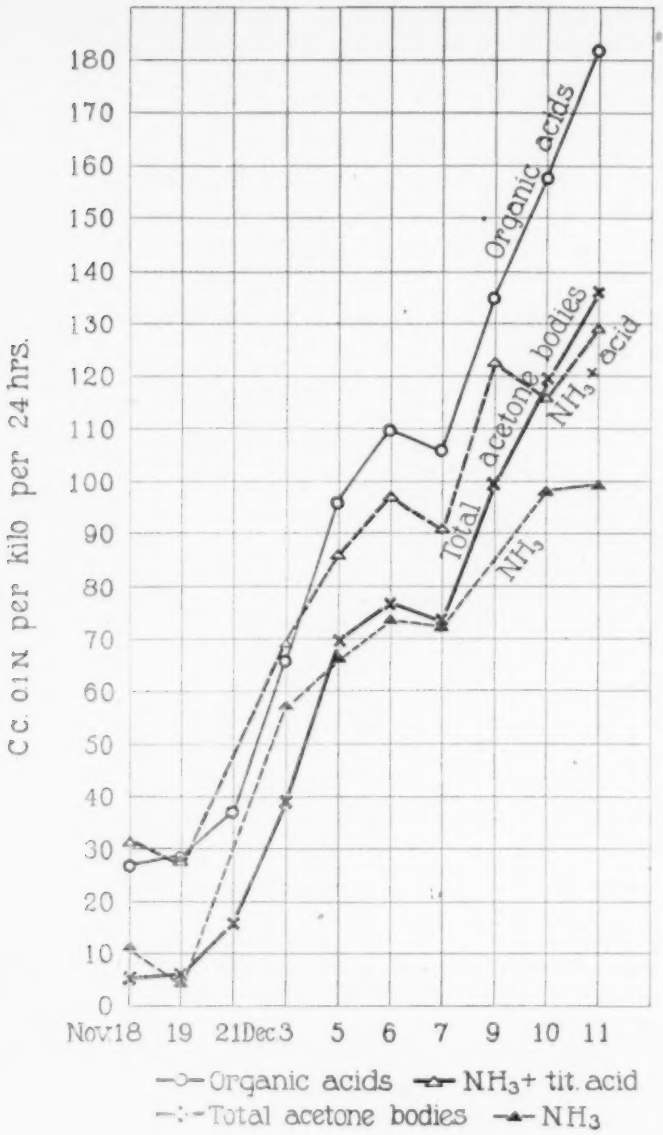

FIG. 1. Excretion in a case of diabetic acidosis.

Comparison of Total Organic Acid Excretion with Acetone Bodies Excretion in Diabetes.

The data given in Fig. 1 were obtained with the only case of diabetic acidosis which we have studied since the organic acid titration method has been available. Although all the data are from one case, they nevertheless represent every stage of diabetic acidosis, from the time when it was slight, with little ketonuria, up to the point of coma, with tremendous ketonuria. The patient was a child of 2 years, weighing 8 kilos. The organic

TABLE X.

Organic Acid Excretion in a Non-Fatal Case of Methyl Alcohol Poisoning.

Date.	CO_2 bound as bicarbonate by 100 cc. of plasma.	Excretion per liter urine.						
		Creatine.	Creatinine.	Total 0.1 N organic acids.*	0.1 N acetone bodies.	0.1 N lactic acid.	0.1 N formic acid.	Undetermined 0.1 N organic acids.
1919	*cc.*	*gm.*	*gm.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Nov. 24.....	36.4	0.202	0.558	2,042		173	274	1,595
" 25.....	36.0	0.283	1.000	2,076	481	83		1,512
" 26.....	86.2	0.535	0.800	1,377	143	30	130	1,074
" 29.....	76.7	0.300	0.590	262				
Dec. 1.....		0.180	0.538	129				
" 2.....		0.137	0.557	141				
" 3.....				220†				
" 11.....		0.105	0.378	86				
" 17.....		0.024	0.476	138				
" 20.....								

* Corrected for creatine and creatinine.

† " " " " " estimated not determined.

acid figures recorded in Fig. 1 are not corrected for creatine and creatinine, so that they are higher than, though parallel to, the actual organic acid excretion. The "total acetone bodies," β-hydroxybutyric acid, acetoacetic acid, and acetone were determined by the gravimetric method of Van Slyke (1917), the ammonia as described by Van Slyke and Cullen, and the titratable acid by the method of Folin (1903).

It is evident from the chart that the organic acids of the urine, determined by the technique outlined above, paralleled the ace-

tone body excretion with a high degree of accuracy through all stages of the acidosis, the parallelism being more accurate than that of the ammonia, or even the ammonia plus titratable acid.

It appears that the rise above the normal output in organic acid excretion may be used as an approximate measure of the acetone body excretion in diabetes, the determination of organic acids being as simple as that of ammonia and less influenced by other factors, such, in particular, as alkali administration.

Organic Acid Excretion in Methyl Alcohol Poisoning.—The data of Table X illustrate an acidosis caused by organic acids other than the familiar acetone bodies. The data represent some preliminary work on methyl alcohol poisoning and are inserted here only for their interest in illustrating a hitherto unfamiliar type of acidosis.

SUMMARY.

The organic acids present both free and as salts in urine are estimated by titrating between the hydrogen ion concentrations represented by pH 8 and pH 2.7 respectively, after removal of phosphates and carbonates by means of calcium hydroxide. It appears that the titration represents between 95 and 100 per cent of the organic acids present. It also includes weak bases whose dissociation constants fall within a range in the neighborhood of 10^{-11}, but of this class only creatinine, and at times creatine, appear to be present in significant amounts in human urine.

The average 24 hour excretion of organic acids in thirteen healthy young men was, per kilo of body weight, 8.2 cc. of 0.1 N acid uncorrected for creatinine, or approximately 6 cc. corrected for creatinine; the extreme range was from 5.7 to 9.8 cc. uncorrected for creatinine. There appears to be little difference between day and night periods in rate of organic acid excretion.

Data from cases of methyl alcohol poisoning and diabetes respectively are given as examples of acidosis due to organic acids of different types. In the case of methyl alcohol poisoning part of the total organic acid excretion was due to formic, lactic, and hydroxybutyric acids, but the greater part to acids of unknown nature.

In the case of diabetes, which progressed to coma, the rise in acetone body excretion was accurately paralleled by the rise in

the titrated organic acids. The parallelism was so close as to indicate the probabilities (1) that organic acids other than the acetone bodies are not excreted in significant amounts in diabetic acidosis, and (2) that the easily performed organic acid titration may be used for approximate estimation of the acetone bodies in diabetic urine.

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DETERMINATION OF THE FIBRIN, GLOBULIN, AND ALBUMIN NITROGEN OF BLOOD PLASMA.

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An investigation into the distribution of the plasma nitrogen, which formed part of a study of the fate of the protein digestion products, led us to develop a technique for the determination of the plasma proteins which gives consistent results and requires no special apparatus. It is based entirely on Kjeldahl determinations, of which the following four are required: (1) total nitrogen of the plasma, (2) fibrin nitrogen, (3) filtrate nitrogen (filtrate, containing albumin and non-protein nitrogen, obtained after precipitating the globulins by half saturation with ammonium sulfate), and (4) non-protein nitrogen.

In the technique finally arrived at the fibrin was precipitated by calcium chloride under definite conditions from plasma containing 0.5 per cent of potassium oxalate, was washed free from other nitrogenous substances, and determined by Kjeldahl.

The globulin was precipitated (together with the fibrin) by the usual half saturation with ammonium sulfate. The nitrogen of the filtrate was determined by Kjeldahl, after removal by distillation of the ammonia of the ammonium sulfate. For the distillation it was found necessary to standardize the conditions accurately in order to make the removal quantitative and at the same time avoid splitting off labile nitrogen from proteins. The non-protein nitrogen was determined on a separate sample of plasma.

The fibrin, globulin, and albumin are calculated as follows:

Fibrin N, determined directly.
Globulin N = Total N − (filtrate N + fibrin N)
Albumin N = Filtrate N − non-protein N

There are two steps in the determination of the albumin and globulin contents of plasma; the first is the separation of the two proteins, or groups of proteins, by precipitation of the globulin with salt, either saturated magnesium sulfate, or half saturated ammonium sulfate. Robertson has reviewed the work on globulin precipitation, and is convinced that ammonium sulfate is the most satisfactory salt for the purpose. We have, therefore, utilized ammonium sulfate precipitation from the start, and since the results have been uniformly consistent have not experimented with other globulin precipitants.

The second step is the determination of the proteins after they have been separated. By different authors this has been done by weighing, by nitrogen determination after dialysis to remove ammonium salts, by the nephelometric method, or by the use of the refractive indices of the proteins as developed by Reiss and by Robertson. The errors inherent in washing and weighing the globulin precipitate are too great to allow accurate results. With nephelometric determinations we have not been able to obtain the desirable degree of accuracy, and a proper refractometer was not available at the time the work was done. We consequently were led to develop a technique in which all the final determinations were made by the Kjeldahl method.

Description of Methods.

Fibrin Determination.

To 5 cc. of plasma, from blood to which 0.5 per cent of potassium oxalate has been added, add 150 cc. of 0.8 per cent NaCl and 5 cc. of a calcium chloride solution containing 2.5 gm. of anhydrous $CaCl_2$ per 100 cc. Allow complete coagulation to occur (10 to 15 minutes) and filter through filter paper. Wash with 0.8 per cent NaCl five times, allowing each washing to remain in contact with fibrin for 10 minutes by closing the outlet of the funnel for that period. Transfer filter paper containing fibrin clot to Kjeldahl flask and add 20 cc. of sulfuric acid, 12 gm. of potassium sulfate, and a crystal of copper sulfate, and determine nitrogen in the usual manner.

Albumin Determination.

Precipitation of Globulin.—To 5 cc. of plasma add 20 cc. of water and 25 cc. of saturated ammonium sulfate solution, allow to stand over night, and filter through a dry filter.

Removal of Sulfate Ammonia.—Place 20 cc. of filtrate (= 2 cc. of plasma) in a 500 cc. Kjeldahl flask, add 300 cc. of 50 per cent alcohol, 3 gm. of MgO (Merck's reagent), and 1 cc. of white mineral oil. Distill until distillate gives a negative test with red litmus paper.

Digestion of Residue.—To residue add 25 cc. of concentrated H_2SO_4, 5 gm. of K_2SO_4 (addition of more, with magnesium sulfate present, would cause bumping), and a small crystal of copper sulfate. Digest to a light brown color. Then wash flask down with a few cc. of water and add 10 cc. more of H_2SO_4. Continue digestion over a low flame for about 3 hours. Distill into N/14 HCl in the usual manner. Calculate nitrogen as "filtrate nitrogen."

Albumin nitrogen = Filtrate nitrogen − non-protein nitrogen

Total Plasma Nitrogen Determination.

The total nitrogen determinations are carried out on 2 cc. of plasma by the regular Gunning-Kjeldahl method, using 20 cc. of concentrated H_2SO_4, 12 gm. of K_2SO_4, about 0.2 gm. of copper sulfate, and digesting 3 hours after clearing.

Non-Protein Nitrogen Determination.

The non-protein nitrogen is determined in the filtrate obtained by precipitation of the plasma protein in 9 volumes of 2.5 per cent trichloroacetic acid (Greenwald, 1915). A 50 or 100 cc. measuring flask is half filled with the trichloroacetic acid solution, to which 5 or 10 cc. of plasma are added. The flask is then filled to the mark with the trichloroacetic acid solution, and the contents are thoroughly mixed. After standing 1 hour the contents of the flask are filtered through a dry filter, the filtrate is measured, and transferred to a Kjeldahl flask. 20 cc. of H_2SO_4, 12 gm. of K_2SO_4, and a crystal of copper sulfate are added, and the nitrogen is determined in the usual manner.

On the basis of Greenwald's recent results (1918), it would seem slightly preferable to use 5 per cent rather than 2.5 per cent trichloroacetic acid, The differences introduced are so minute, however, that for the determination of the proteins they are not significant.

Correction for Reagents.

It is necessary to determine the corrections for all the reagents. Our blanks averaged 0.46 cc. of N/14 HCl, a rather high value, but one constant for the given lot of reagents.

EXPERIMENTAL.

Determination of Filtrate Nitrogen.

In order to determine the albumin and non-protein plasma nitrogen in the filtrate from the globulin, it was necessary to find conditions for distilling off the ammonia of the ammonium sulfate without splitting off ammonia from any of the plasma proteins. In order to avoid such decomposition it was desirable to use in the distillation as weak an alkali as possible. Magnesium oxide, in former work on protein analyses (Van Slyke), had been found to be as mild an alkali as could be successfully used to drive off ammonia, and it proved to be suitable in this case also when used together with alcohol. It was found that the physical properties of the oxide were of importance. Tremendous bumping, resulting in broken flasks, took place with all but one brand of MgO. When Merck's reagent oxide was used with the addition of 1 cc. of white mineral oil, and the flask with its contents was frequently shaken until the boiling commenced, the distillation proceeded smoothly and without bumping.

Distillation with Water.—2 cc. of plasma and 10 cc. of saturated ammonium sulfate solution were diluted with 200 cc. of water in a 500 cc. Kjeldahl flask and an excess of magnesium oxide, 2 to 3 gm., was added (at 20°C. a half saturated ammonium sulfate solution contains 38 gm. per 100 cc. of the solution; 10 cc. would then require 1.7 gm. of MgO). The water and ammonia were distilled off. The nitrogen in the residue was then determined as outlined below.

In each case distillation was continued until moistened red litmus paper held in the distillate no longer turned blue at once. Actual cessation of ammonia distillation did not occur, because of a slight but continuous splitting off of ammonia from the proteins. Consequently if the litmus paper was held in the distillate for 2 minutes, an alkaline reaction could be obtained at any stage of the distillation. The end-point was therefore taken as the stage at which the distillate failed to turn litmus at once.

It was found that frequently the ammonia was not completely removed by distilling nearly to dryness once; it was necessary

TABLE I.

Albumin Determination. Removal of Ammonium Sulfate Nitrogen by Water Distillation in Presence of Magnesium Oxide.

2 cc. plasma + 10 cc. saturated $(NH_4)_2 SO_4$ solution. Total nitrogen of plasma controlled on 2 cc. duplicates.

Method of concentration.	MgO	H_2O used.	Final volume.	Proportion of plasma nitrogen recovered.
	gm.	*cc.*	*cc.*	*per cent*
Distillation from Kjeldahl flask.	2.3	400 in two portions.	50	98.3
" " " "	2.3	400 " " "	50	97.8
" " " "	2.3	400 " " "	50	96.0
" " " "	2.3	400 " " "	50	97.8
" " " "	2.3	400 " " "	50	96.0
" " " "	2.3	400 " " "	50	96.0
" " " "	2.3	400 " " "	50	93.0
" " " "	2.3	400 " " "	50	94.0
" " " "	2.3	400 " " "	50	92.0

to add a second 200 cc. of water and distill again. With two distillations all the sulfate ammonia was removed, but with it from 2 to 8 per cent of the plasma nitrogen was lost, apparently as the result of ammoniacal decomposition of the plasma proteins (Table I). Regulation of the rate of distillation, of the final volume, *etc.*, all failed to prevent this loss.

Distillation with Alcohol.—In attempting to reduce both time and temperature of distillation, a mixture of alcohol and water was substituted for the water. A few of the results are given in Table II.

It is evident that with the use of 50 per cent alcohol, 99 per cent of the plasma nitrogen can be consistently recovered. One distillation only, taking 30 to 45 minutes, is required to drive off all ammonia. In studying the distribution of the plasma proteins following digestion, the method was tested several times on plasma from each of a dozen dogs. Between 99.8 and 98.5 per cent of the plasma nitrogen was invariably recovered.

TABLE II

Albumin Determination. Removal of Ammonium Sulfate Nitrogen by Distillation in Presence of MgO and Alcohol.

2 cc. plasma + 10 cc. saturated $(NH_4)_2 SO_4$ solution.

MgO	H_2O	95 per cent alcohol.	Final volume.	Proportion of plasma nitrogen recovered.
gm.	*cc.*	*cc.*	*cc.*	*per cent*
3.5	150	150	25	99.6
3.0	150	150	25	99.2
2.5	150	150	15	99.6
3.0	150	150	Dry.	97.9
3.0	150	150	25	99.0
3.0	150	150	25	99.6
3.0	150	150	25	99.1
3.0	150	150	25	99.2
3.0	150	150	25	99.0

Fibrin Determination.

Dilution of Plasma.—If calcium is added to undiluted oxalated plasma, the entire mass jellies. If, however, the plasma is diluted with isotonic NaCl solution, the fibrin forms as a delicate membrane which contracts upon shaking or stirring to a small compact mass. Moreover, if the plasma is diluted ten- to thirtyfold the quantity of nitrogen in the solution adhering to the small clot is presumably much smaller than if the fibrin is whipped from undiluted plasma. In order to ascertain the best dilution, oxalated plasma was mixed with varying amounts of isotonic NaCl solution, which, to prevent globulin precipitation, was used instead of water. Preliminary experiments had shown that at least 2 molecules of calcium chloride should be added for each molecule of potassium oxalate present in the plasma.

The time required to complete the formation of the fibrin clot and the appearance of the clot were noted. Typical results are shown in Table III.

The clots formed when the plasma was diluted with 10 volumes of salt solution did not appear so satisfactory as those formed with either 20 or 30 volumes. There was no choice between

TABLE III.

Determination of Dilution Most Suitable for Plasma Fibrin Coagulation.

1 cc. plasma diluted as indicated.

Plasma.		0.8 per cent NaCl.	Calcium added for recalcification.			Time required for complete clotting.	Coagulation observed.
No.	Oxalate concentration.		$CaCl_2$ solution.		Oxalate equivalent.		
	per cent	*cc.*	*per cent*	*cc.*		*min.*	
1	0.25	5	2.5	1	15		
	0.25	10	2.5	1	15	9	Not complete
	0.25	20	2.5	1	15	5	Good.
	0.25	30	2.5	1	15	6	"
	0.25	40	2.5	1	15	7	
2	0.25	10	0.17	2	2	8.5	
	0.25	20	0.17	2	2	8.5	Good.
	0.25	30	0.17	2	2	9	
	0.25	10	0.17	4	4	7	
	0.25	20	0.17	4	4	9.5	
	0.25	30	0.17	4	4	9	Good.
	0.25	10	2.5	1	15	7	
	0.25	20	2.5	1	15	8.5	Good.
	0.25	30	2.5	1	15	9	
1	1.0	10	0.48	5	15	7	
	1.0	20	0.48	5	15	7	
	1.0	30	0.48	5	15	7	
	1.0	40	0.48	5	15	21	

20 and 30 volumes, but with 40 volumes the clot appeared less satisfactory, and the time required for its formation was greater than with 20 or 30 volumes. The use of either 20 or 30 volumes of 0.8 per cent salt solution was, therefore, adopted.

Permissible Range of Calcium Chloride Concentration.—In order to determine the range of calcium concentration over which

satisfactory fibrin clot formation can occur, varying amounts of calcium chloride were added to a series of tubes each containing 1 cc. of plasma and 20 cc. of salt solution.

It is evident from Table IV that fibrin formation is complete if calcium is added in from two to twenty equivalents of the oxalate present. Allowing for a maximum oxalate concentration of 1 per cent, each cc. of plasma would require for an equivalent amount

TABLE IV.

Influence of Concentration of Calcium Chloride on Fibrin Clot Formation.

1 cc. plasma + 20 cc. 0.8 per cent NaCl recalcified in the presence of varying amounts of oxalate.

Oxalate concentration.	Calcium added for recalcification.			Time required for complete clotting.
	$CaCl_2$ solution.		Oxalate equivalent.	
per cent	*per cent*	*cc.*		*min.*
1	0.5	0.5	0.37	∞
1	0.5	1	0.75	∞
1	0.5	2	1.5	9.0
1	0.5	4	3.0	5.0
1	2.5	1	3.7	5.5
1	2.5	2	7.5	6.5
1	2.5	4	15.0	11.0
0.25	2.5	0.25	3.7	7.0
0.25	2.5	0.5	7.5	7.5
0.25	2.5	1.0	15.0	7.5
0.25	2.5	1.5	22.5	9.0
0.25	2.5	2.0	30.0	>15
0.25	0.17	0.5	0.5	>30
0.25	0.17	1	1	>30
0.25	0.17	2	2	9
0.25	0.17	4	4	9

of calcium 0.2 cc. of 2.5 per cent $CaCl_2$. 1 cc. of 2.5 per cent $CaCl_2$ solution would then contain five equivalents. If the oxalate concentration were 0.25 per cent, which is just sufficient to prevent coagulation, the use of an equal volume of 2.5 per cent $CaCl_2$ would mean that twenty equivalents of calcium had been used. This amount of $CaCl_2$ does not interfere with the clot formation, and provides for all probable concentrations of oxalate.

Collection, Washing, and Nitrogen Determination of the Fibrin.— It has been our experience that the determination of the nitrogen content of a protein is a more accurate measure of its mass than the method of drying to constant weight. The errors due to adherent substances or to partial decomposition during drying are thus eliminated. After testing varying conditions for the Kjeldahl digestion the following procedure was adopted. The filter paper with the washed fibrin clot was transferred to a 500 cc. Kjeldahl flask. 20 cc. of concentrated sulfuric acid, 12 gm. of potassium sulfate, and a small crystal of copper sulfate were added, and the mixture was digested for 3 hours after the clearing of the solution.

TABLE V.

Quantitative Test of Fibrin Method.

5 cc. plasma. 0.25 oxalate; 0.8 per cent NaCl solution, $CaCl_2$, and washing as indicated. Nitrogen determined by Kjeldahl method.

H_2O	0.8 per cent NaCl added.	Calcium added for recalcification.			Washed.	Fibrin N. per 100 cc.
		$CaCl_2$ solution.		Oxalate equivalent.		
cc.	*cc.*	*per cent*	*cc.*			*gm.*
	100	2.5	5	15	0.8 per cent NaCl five times.	0.046
	150	2.5	5	15	0.8 " " " " "	0.044
	150	2.5	10	32	0.8 " " " " "	0.044
	150	1.0	5	6	0.8 " " " " "	0.042
	150	1.0	10	12	0.8 " " " " "	0.044
	50	1.0	10	12	0.8 " " " " "	0.044
	150	1.0	10	12	H_2O till chloride-free.	0.042
	50	1.0	10	12	" " "	0.048
100		1.0	10	12	" " "	0.048

The fibrin clot was collected by filtration. In order to be sure that all traces of soluble proteins were removed from the clot, experiments were done to determine whether or not washing with NaCl only or with water was more satisfactory. Each washing was allowed to remain in contact with the clot for several minutes to allow time for diffusion. This was done by closing the outlet at the bottom of the funnel stem for the desired time.

It is evident from Tables V and VI that five washings with salt solution or washing with water until the filtrate is salt-free yields consistent results.

TABLE VI.

Test of Different Methods of Washing Fibrin Clot.

5 cc. plasma diluted to 150 cc. with 0.8 per cent NaCl, 5 cc. 2.5 per cent $CaCl_2$ solution added. Fibrin washed on filter paper as indicated. Nitrogen determined by Kjeldahl method.

0.8 per cent NaCl.	H_2O	Each washing allowed to stand.	Fibrin N per 100 cc.
		min.	*gm.*
Five times.		10	0.077
" "		10	0.075
" "		10	0.073
" "		15	0.075
" "		30	0.079
	Five times.	30	0.075
	" "	30	0.075
	Three "	30	0.083
	Four "	30	0.075
	" "	60	0.071

TABLE VII.

Influence of Excess of Decalcifying Salts on the Concentration of Total Nitrogen and Fibrin in Plasma.

Blood was drawn mixed with sufficient oxalate to prevent clotting. Salt was then added to portions of blood to concentration indicated. Blood centrifuged and plasma analyzed in usual way.

Sample No.	Concentration of oxalate.	Total N per 100 cc.	Fibrin N per 100 cc.
	per cent	*gm.*	*gm.*
1	0.1	1.16	0.024
	1.1	1.018	0.029
	2.0	0.958	0.025
2	0.5	1.0	0.049
	1.0	0.929	0.051
	2.0	0.874	0.051
3	0.5	0.898	0.051
	1.0	0.862	0.047
	2.0	0.746	
To plasma from 0.5 per cent oxalated blood (Sample 3) oxalate was added to make 2 and 5 per cent.	2.0	0.887	
	5.0	0.887	

Influence of the Oxalate Concentration of the Plasma.—It was found (Table VII) that the amount of oxalate present in the plasma had no effect on the fibrin, but that variation in the concentration of oxalate in the whole blood before centrifugation has a considerable effect on the total nitrogen content of the plasma, presumably because of effects on the water distribution between cells and plasma. Consequently in order to obtain accurately comparable results on different plasmas a constant concentration of oxalate must be used. We add 0.5 gm. of potassium oxalate per 100 cc. of whole blood.

SUMMARY.

Methods are described for separation of the fibrin, globulin, and albumin of blood plasma in such a manner that they may be determined by the Kjeldahl method.

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STUDIES OF VARIATIONS IN THE CHEMICAL COMPOSITION OF HUMAN BLOOD.

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INTRODUCTION.

Studies on the chemical composition of human blood have been largely confined to the determination of the nature and the amounts of the constituents. The more exact methods of Folin (1, 2) and his collaborators have placed in the hands of the investigator a means of studying the changes taking place in the concentration of the various blood components in health and disease. The early results have been amplified and extended so that today the literature contains many reports of the normal limits within which these substances fluctuate. Probably the most complete survey of the chemical and physical composition of human blood is that published by Gettler and Baker (3). Studies which have been made of the relations between the determinable nitrogenous constituents have been limited in their applicability by the omission of one or more of the soluble nitrogenous components from direct estimation. The extensive studies of Bang (4) and Feigl (5) are more recent examples of this failure to consider the individual components.

In view of this lack of tangible data a study is here made of the relative variability and reciprocal relations of the soluble nitrogenous compounds more commonly associated with protein metabolism together with the total nitrogen and the sugar of the blood. The data naturally divide under four headings. There is the study of the relative variability of the blood components for the individual; the discussion of the relative variability of these constituents in a group series of bloods; the influence of the time of withdrawal of the blood on its composition; and lastly, the study of the changes in the amounts, distribution, and interrelations of the nitrogen of the various constituents which accompany the changes in the level of the total non-protein nitrogen itself.

Methods.

The individuals from whom the bloods were taken for these studies were patients and nurses at this hospital. No subject was accepted for study who presented evidence of metabolic disorder. The use of patients suffering from one sort or another of mental disorder for a study of this nature seems to be allowable on the basis of the early findings of Folin (6) that the urinary nitrogenous constituents of such patients are within normal limits. Tracy and Clark (7), however, express the opinion that certain individuals of this type may present some alterations in creatinine excretion due to their relative muscular inactivity.

No quantitative dietary regulation was attempted since the general uniformity of the hospital diet from a qualitative point of view would tend towards the establishment of a qualitative uniformity in its manner of utilization, the degree of which would be dependent ultimately upon the amount of and the individual reaction to the ingested foodstuffs. Any regular variation tendencies that might be found would be all the more indicative of an underlying constancy of metabolic relation. The limits to which data from such material can be used are well recognized and the interpretations that are read into the results are consequently subject to similar conditions and are to be taken with that understanding.

Since the investigations of Raiziss, Dubin, and Ringer (8) indirectly, and of Addis and Watanabe (9), Gettler and Baker (3), and others directly support the contention that a rigid level of content of blood constituents is obtainable only with difficulty and after a considerable period of regulated food intake, the samples of blood to be analyzed were taken at the uniform hour of 11 a.m., $3\frac{1}{2}$ hours after the first meal, unless otherwise noted. The blood from any single individual was not taken more often than once in 7 days.

The blood was drawn into a Record syringe containing a small amount of potassium oxalate (1). Care was taken to avoid the sucking in of air, and after filling the syringe the point of the needle was plunged under paraffin oil (10), and 2 cc. of blood were expressed. This part of the sample was used for the determination of the alkaline reserve. The remainder of the blood, about 25 to 30 cc. in amount, was transferred to a small bottle containing oxalate crystals and brought immediately to the laboratory. From this sample 1 cc. was removed with an Ostwald pipette and diluted to 50 cc. with distilled water in a graduated flask. 1 cc. portions of the diluted blood were used for the estimation of the total nitrogen according to the method of Folin and Farmer (11). The residual sample was freed from protein by the procedure of Folin and Wu (12), and the non-protein nitrogen, urea nitrogen, creatinine, creatine, uric acid, and sugar were determined by following the methods outlined in their publication. The standard for the creatinine and creatine determinations was pure creatinine prepared by the method of Benedict (13). The picric acid was purified according to Folin and Doisy (14), solutions of this substance being made up fresh each day. In the aerations the turpentine and resin mixture of Kendall (15) was used as a foam breaker with good results. The only difficulty that presents itself with this reagent was that there remains a considerable residuum of resinous matter in the test-tubes which must be removed after each total nitrogen determination by boiling with $CuSO_4$-K_2SO_4-H_2SO_4 mixture. The amino-acid nitrogen was determined in aliquots of the filtrate after evaporation to a small volume with a few drops of carbonate as indicated by Whipple and Van Slyke (16), the analysis being made with the micro apparatus and technique of the latter investigator (17).

Preliminary tests showed that the variations in the results obtained in these filtrates from results obtained in the filtrates from other reliable methods of precipitation were negligible. Repeated tests failed to show any marked interfering factor arising from the action of the nitrous acid on the urea during the analysis as carried out and hence no correction was made for this substance. The residual or rest nitrogen was obtained as the difference between the sum of the nitrogen found as urea, creatinine, creatine, uric acid, and amino-acids and that of the total non-protein nitrogen. Separate ammonia determinations were not made. The extreme lability of this substance (18), the observations of Gad-Andersen indicating that the increase in blood ammonia on standing may be due to a concurrent urea decomposition (19), and the confirmation of Folin's (20) statement that the amounts of ammonia in these filtrates are so small as to be negligible, made any attempts at its determination here superfluous.

Individual Variability.

Table I presents the figures for the amounts and the average deviations of each constituent determined in the bloods of nine individuals. The specimens from each individual were taken at weekly intervals 3½ hours after the morning meal, with the exception that Sets 7′, 8′, and 9′ were taken before breakfast. These latter sets are from the same subjects as are the figures in Sets 7, 8, and 9. The results are representative of amounts of nitrogen per 100 cc. of blood.

It will be noted that while the composition of the blood in any given individual varies from week to week the level of the concentrations of the various substances is individual in character.

In Table II are given the sums of the average deviations for each individual. The marked differences observed are taken as meaning differences in metabolic stability.

In order to study the relative variability of the constituents the average deviations were in the case of each blood arranged in the order of increasing value. Since nine constituents are reported there are nine possible places on the scale of ascending variability into which any one of the constituents might fall. Table III gives for each constituent its relative place in the scale of increasing variability in terms of the per cent incidence.

TABLE I.

Individual Variations in Blood Composition.

Set No.	Total N.	Non-protein N	Urea N.	Creatinine N.	Creatine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	2.6	38.1	21.4	0.48	1.16	0.93	4.1	10.0	88
	3.1	30.6	12.5	0.48	1.35	1.10	6.7	8.5	109
	2.32	30.6	10.6	0.52	1.31	0.77	5.6	11.8	106
	3.06	36.3	17.5	0.44	1.22	1.03	4.8	11.3	105
	3.21	37.8	18.2	0.48	1.15	0.83	4.7	12.4	100
	2.65	31.5	17.6	0.44	1.15	0.70	5.0	6.4	100
Average...	2.83	34.1	16.3	0.47	1.22	0.89	5.1	10.1	101
" deviation	10.6	9.6	19.4	5.0	5.8	14.3	12.8	17.5	5.3
2	2.75	39.0	20.1	0.48	1.22	0.90	4.3	12.0	90
	2.22	39.9	15.1	0.52	1.47	0.70	5.3	16.8	116
	2.91	33.6	17.7	0.44	0.96	0.73	5.7	8.1	92
	2.56	34.8	15.0	0.48	1.52	0.70	4.6	12.5	101
	2.71	33.0	15.4	0.48	1.19	0.70	5.4	9.8	96
	2.80	39.0	21.1	0.48	1.22	0.96	4.5	10.7	97
Average...	2.66	36.5	17.4	0.48	1.26	0.78	4.9	11.6	99
" deviation	6.7	7.5	13.5	2.8	12.2	12.7	10.2	18.3	6.7
3	2.67	43.8	25.1	0.41	1.35	0.77	4.9	11.3	135
	3.09	32.4	15.5	0.48	1.41	0.53	4.8	10.1	120
	2.94	42.9	24.8	0.44	1.22	0.73	6.2	9.5	113
	3.57	41.4	19.5	0.48	1.44	0.70	6.3	13.0	134
Average...	3.09	40.1	21.1	0.45	1.35	0.68	5.5	11.0	125
" deviation	8.5	9.5	18.0	6.1	5.1	11.3	12.7	10.6	7.1
4	3.52	43.3	20.1	0.44	1.25	0.70	4.5	16.2	90
	2.61	38.7	21.2	0.52	1.47	0.73	3.4	11.4	120
	2.61	34.8	15.5	0.41	1.99	0.85	3.6	13.5	87
	2.65	38.4	18.5	0.44	1.78	0.87	3.9	12.9	115
	2.71	37.5	15.9	0.48	1.41	0.83	4.8	14.1	97
	2.80	36.6	22.0	0.41	1.44	0.80	5.4	6.6	100
Average...	2.82	38.2	18.9	0.45	1.39	0.80	4.3	12.4	101
" deviation	8.4	4.8	11.8	7.4	13.0	6.6	14.7	18.7	10.4

TABLE I—*Continued.*

Set No.	Total N.	Non-protein N.	Urea N.	Creatinine N.	Creatine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
5	3.24	46.7	13.1	0.41	1.70	0.93	4.6	26.0	105
	2.64	35.4	18.1	0.44	1.35	0.90	5.0	9.3	112
	3.00	34.5	17.7	0.48	1.31	1.07	5.1	8.8	104
	3.00	36.6	13.9	0.44	1.25	1.27	5.7	14.0	126
Average...	2.97	38.3	15.7	0.44	1.40	1.04	5.1	14.5	112
" deviation	5.7	10.9	14.0	3.9	10.5	12.3	5.9	39.5	6.4
6	3.26	40.0	22.6	0.43	1.71	0.57	5.9	8.8	125
	3.30	34.5	17.7	0.40	1.45	0.61	5.6	8.8	122
	2.73	37.3	19.2	0.44	1.44	0.56	6.0	9.6	166
	2.73	33.7	16.7	0.48	1.48	0.73	6.6	7.8	145
Average...	3.00	36.4	19.0	0.44	1.52	0.62	6.0	8.8	139
" deviation	9.2	6.3	9.9	5.1	6.2	9.8	4.8	5.0	11.5
7	3.03	53.6	39.7	0.57	1.42	1.98	6.7	9.4	108
	3.03	55.6	39.0	0.56	1.50	2.14	6.8	10.2	99
	3.00	52.6	37.5	0.62	1.40	2.14	6.4	8.6	92
	3.09	54.5	31.9	0.56	1.27	2.45	6.1	22.4	99
Average...	3.04	54.6	37.0	0.58	1.40	2.18	6.4	12.6	99
" deviation	0.90	1.8	6.9	3.8	4.5	6.3	3.9	38.8	4.0
8	3.33	33.7	16.6	0.46	1.20	0.79	6.1	8.6	89
	3.12	33.1	15.9	0.48	1.42	0.79	3.6	11.0	133
	3.30	34.6	13.1	0.43	1.19	0.70	5.3	13.9	96
	3.42	38.7	16.9	0.50	1.25	0.96	5.0	14.1	115
Average...	3.29	35.0	15.6	0.47	1.26	0.81	5.0	11.9	108
" deviation	2.6	5.2	8.0	4.8	6.0	9.3	13.9	17.7	14.8
9	3.66	35.9	23.6	0.54	1.49	0.69	6.9	3.7	114
	3.15	45.5	23.8	0.54	1.49	0.82	6.3	12.5	106
	3.00	40.0	22.1	0.53	1.30	0.99	5.3	9.8	103
	2.88	37.3	21.3	0.53	1.38	0.80	5.7	7.6	111
Average...	3.17	39.7	22.7	0.53	1.41	0.82	5.8	8.4	108
" deviation	7.7	7.6	4.3	1.0	5.2	9.8	5.0	32.8	3.7

TABLE I—*Concluded.*

Set No.	Total N.	Non-protein N.	Urea N.	Creatinine N.	Creatine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
7′	2.88	56.6	33.0	0.53	1.48	2.03	5.9	13.7	119
	3.30	52.2	31.9	0.52	1.51	2.00	5.6	10.6	116
	2.97	50.9	30.0	0.62	1.35	2.28	6.5	10.1	87
	3.00	54.5	30.6	0.53	1.31	1.74	5.6	14.7	118
Average...	3.04	53.5	31.4	0.55	1.41	2.01	5.8	12.3	110
" deviation	4.3	3.7	3.3	6.8	5.8	7.2	2.4	15.5	10.3
8′	3.30	32.8	14.2	0.46	1.28	0.78	5.0	11.0	100
	3.45	31.4	16.7	0.46	1.40	0.73	5.1	7.1	105
	3.24	37.1	14.2	0.48	1.21	0.66	5.6	14.9	104
	3.16	36.2	14.8	0.48	1.31	0.67	5.4	13.6	108
Average...	3.29	34.4	15.0	0.47	1.30	0.71	5.3	11.7	103
" deviation	2.6	6.6	5.9	2.0	2.9	6.3	4.2	14.6	2.5
9′	3.33	36.2	19.8	0.49	1.20	0.76	4.8	9.1	138
	3.00	32.9	18.9	0.45	1.46	0.58	4.9	7.5	126
	3.33	36.7	22.6	0.48	1.46	0.51	6.3	5.1	97
	3.00	30.6	19.1	0.48	1.31	0.57	5.7	3.4	96
Average...	3.16	34.1	20.1	0.47	1.36	0.60	5.4	6.2	114
" deviation	5.2	6.8	6.2	3.1	7.5	12.4	10.6	32.2	15.5

TABLE II.

Relative Metabolic Stability of Different Individuals as Calculated from Average Deviations Observed in Table I.

Set No.	1	2	3	4	5	6	7	8	9	7′	8′	9′
Relative stability	100	91	89	96	109	68	71	82	77	59	48	101

Table III reveals a quite consistent tendency to increasing variability of the constituents in the order given, the scatterings that occur being due in large part to individual differences in nature of metabolic stability.

Group Variability.

We turn now from the consideration of the variations in the constituents of the blood of an individual to a study of the variations within a group of bloods. Although the literature contains many reports and controversies concerning the limits that may be considered as normal for the various substances determined, it is not the purpose of this study to enter into any discussion of the relative merits of the results of the various investigators or to set new limits or to confirm old ones. That no hard and fast lines can be drawn for the limits within which the amounts of any individual component may be supposed to fall to

TABLE III.

Order of Variability of Blood Constituents in the Individual.

	1	2	3	4	5	6	7	8	9
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Creatinine N	50	25	17			8			
Total N	17	25	8	25	8	8	8		
Non-protein N	8	8	17	17	17	25		8	
Creatine N	8		8	25	33	17	8		
Sugar		25	8	8	17			25	17
Amino-acid N	17		8	17	8	17	17	17	
Uric acid N		8			8	8	58	17	
Urea N		8	17		8	17	8	25	17
Rest N			17			8		8	67

be considered normal in amount is amply evidenced by the studies of Addis and Watanabe (21), McLean and Selling (22), and others. The conservative opinion seems to be that except in extreme cases it is the blood picture as a whole that is indicative of a pathological condition, rather than the amount of any particular constituent which for the moment may be exhibiting an abnormality that may be more apparent than real.

In order to save space and repetition Table IV is limited to the figures representing the range within which the various substances fluctuate, the averages of the amounts found, the average deviation for each constituent, and the relative variability. The absolute values are given in terms of nitrogen per 100 cc. of blood and are arranged in the order of their increasing variability.

In general it can be said that a comparison of the range of variation observed in these results with the reported findings of other workers shows a general agreement in values. It is to be remembered that the figures represent the extreme limits found and that where they appear to differ materially from the so called normal limits they nevertheless may properly be considered as within what might be expected in the sense of the theory of probability.

An inspection of the average deviations makes evident that they are not simply related to the absolute amounts of the respective substances but rather express differences in the susceptibility of the constituents to variation. A comparison of the

TABLE IV.

Range of Variation, Average Amounts, Average Deviations, and Relative Variability of the Blood Constituents Determined.

	Range.	Average.	Average deviation.	Relative variability.
	mg.	*mg.*	*per cent*	
Creatinine N	0.37–0.60	0.47	7.3	100
Non-protein N	27.3–45.5	35.6	9.1	125
Total N	2560–4290	307.0	9.4	129
Creatine N	0.62–1.78	1.31	11.5	158
Sugar	85–166	112.0	13.7	188
Uric acid N	0.50–1.16	0.78	14.9	204
Amino-acid N	3.1–7.2	4.9	15.3	210
Urea N	9.7–25.1	17.1	16.6	227
Rest N	3.7–18.3	11.1	20.1	275

order of variation here obtained with that shown in Table III demonstrates that no significant changes have occurred, the interchange of position of the total nitrogen and the non-protein nitrogen, as well as that occurring in the amino-acid and uric acid nitrogen, being due to quantitatively negligible differences.

Such being the case we have an interesting sequence in variability of the constituents under investigation. The causes of these differences in variability cannot be attributed solely to differences in amounts or nature of absorbed material since the order of variability tends to be the same in different individuals presumably ingesting quantitatively different diets. Nor can these differences be wholly ascribed to individual differences in

metabolic stability. It would appear as if the order of variability here manifested is rather the expression of an underlying uniformity of metabolic mechanism specific for the species.

The significance of the position of the various constituents in Table IV is not wholly obvious. They can be, however, divided roughly into three groups, one of relatively low variability containing the creatinine, the total non-protein nitrogen, and the total nitrogen; one of intermediate variability, into which fall the creatine and sugar; and one of relatively high variability containing the uric acid, amino-acid, and urea nitrogen.

The fact that the creatinine appears to be the least susceptible to variation would lend additional support to the conception of Folin and Denis (23) that this substance is the result of a uniform endogenous process and that it represents a certain particular form of protein metabolism.

The relatively low variability of the total non-protein nitrogen is in part accounted for by compensatory variations in deviation occurring between the urea nitrogen, the amino-acid, and rest nitrogen. The variability of the total nitrogen is probably due as much to differences in water intake as to individual differences (24).

The differences in the stability of the metabolic factors leading to the production of creatinine and creatine are well emphasized in the 60 per cent greater variability of the latter.

The factors concerned in the regulation of the amounts of sugar in the blood have been quite fully discussed in a recent publication by Grote (25). It is evident from Table IV that the sugar variability lies near the middle of the scale.

The relatively high variability of the uric acid is probably due to variations in the activity of the endogenous processes giving rise to this blood constituent. According to Mareš (26) these processes are largely confined to the nuclei of the digestive glands, but recent work of Lewis, Dunn, and Doisy (27) has shown that there is a general nuclear activity induced by a stimulative effect of the amino-acids. That the source of the variability is endogenous is strengthened by the findings of Denis (28) that the concentration of uric acid in the blood does not rise in normal individuals during the ingestion of purine-containing foods.

Both Folin and Denis (29) and Van Slyke and Meyer (30) have shown the existence of an interrelation between the metabolic factors concerned with the concentration of amino-acids and urea in the blood, and in view of our present ideas concerning their metabolism a relatively high variability in their concentration is not unexpected. The rest nitrogen will be considered later.

Influence of Time of Withdrawal of Blood on Its Composition.

Although Gettler and Baker (3) state that the concentrations of the various components of the blood fall within normal limits

TABLE V.

Differences in Concentration of Total Nitrogen, Non-Protein Nitrogenous Constituents, and Sugar in Bloods Taken before and after Breakfast.

	Total N.	Non-protein N.	Urea N.	Creatinine N.	Creatine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
				Sets 7–7′.					
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Before	3.04	53.5	31.4	0.55	1.41	2.01	5.8	12.3	110
After	3.04	54.6	37.0	0.58	1.40	2.18	6.4	12.6	99
				Sets 8–8′.					
Before	3.29	34.4	15.0	0.47	1.30	0.71	5.3	11.7	103
After	3.29	35.0	15.6	0.47	1.26	0.81	5.0	11.9	108
				Sets 9–9′.					
Before	3.17	34.1	20.1	0.47	1.36	0.60	5.4	6.2	114
After	3.17	39.7	22.7	0.53	1.41	0.82	5.8	8.4	108

whether the blood is taken before or 3 hours after breakfast, and Addis and Watanabe (21) confirm this for urea, it is advisable to make a detailed comparison of bloods taken from the same individuals before and 3½ hours after breakfast for obvious practical reasons. In Table I Sets 7, 8, and 9 are from bloods taken at weekly intervals 3½ hours after the morning meal. Sets 7′, 8′, and 9′ are bloods from the same individuals in the order given, taken at weekly intervals before breakfast. To facilitate comparison Table V presents the data in a condensed form. The absolute amounts are the averages for the four weekly determinations on each individual.

It is seen that there is no practical difference in the concentration of the various constituents determined in blood taken 14 and 3½ hours after eating. From the metabolic point of view the blood taken before breakfast shows evidence of a slightly lower metabolism at this period. The non-protein nitrogen, the urea nitrogen, and the uric acid nitrogen are consistently lower, but to a very small extent. It is seen that, as others have shown in similar comparisons, the sugar may be somewhat higher after the longer fast (31). The changes in the average deviations are generally indeterminate.

Nitrogen Distribution in the Blood.

Our present conception of protein metabolism is largely derived from the studies of Folin (32) on the laws governing the chemical composition of the urine. These studies are based on the differences in the nitrogen and sulfur distribution in urines of high and low total nitrogen content. The sharp differences observed in the nitrogen distribution in such urines, however, cannot be expected in the blood. There is, however, a definite tendency for a change in nitrogen distribution to take place with changes in the level of the total non-protein nitrogen. In Table VI are given the analyses previously summarized in Table IV, as nitrogen per 100 cc. of blood, and the per cent of nitrogen of each constituent in terms of the total non-protein nitrogen. The values for the individual samples of blood have been arranged in the order of descending values of the non-protein nitrogen.

With a decrease in the value of the total non-protein nitrogen there is a general tendency for a simultaneous decrease in the absolute amounts of the urea, creatine, uric acid, and amino-acid nitrogen, of which the urea reduction is the most uniform. There are differences in the degree to which these constituents decrease with the non-protein nitrogen, the urea showing relatively the greater diminution. The rest nitrogen seems to be less affected by a change in the non-protein nitrogen level.

The lack of definite change in the concentration of creatinine nitrogen is significant in that the concentration is thus shown to be independent of that of the total non-protein nitrogen.

TABLE VI.

The Amounts and Percentages in Terms of Non-Protein Nitrogen of the Non-Protein Nitrogenous Constituents of 100 Cc. of Human Blood.

No.	Non-protein N.	Urea N.		Creatinine N.		Creatine N.		Uric acid N.		Amino-acid N.		Rest N.	
	mg.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	45.5	23.8	52.4	0.54	1.2	1.49	3.3	0.82	1.8	6.3	13.8	12.5	27.6
2	43.8	25.1	57.3	0.41	0.9	1.35	3.2	0.77	1.8	4.9	11.2	11.3	25.8
3	43.2	20.1	46.5	0.44	1.0	1.25	2.9	0.70	1.6	4.5	10.4	16.2	37.5
4	42.9	24.8	57.8	0.44	1.0	1.22	2.8	0.73	1.7	6.2	14.5	9.5	22.1
5	42.3	20.0	47.3	0.52	1.2	1.25	3.2	0.77	1.8	5.4	12.8	14.2	33.6
6	41.4	19.5	47.1	0.48	1.2	1.44	3.5	0.70	1.7	6.3	15.2	13.0	31.4
7	40.5	15.6	38.5	0.44	1.1	1.57	3.9	0.77	1.9	3.9	9.6	18.3	45.2
8	40.0	22.1	55.2	0.53	1.3	1.30	3.3	0.99	2.5	5.4	13.3	9.8	24.5
9	40.0	22.6	56.4	0.43	1.1	1.71	4.3	0.57	1.4	5.9	14.8	8.8	22.0
10	39.0	20.1	51.5	0.48	1.2	1.22	3.1	0.90	2.3	4.3	11.0	12.0	30.7
11	39.0	21.1	54.1	0.48	1.2	1.22	3.1	0.96	2.5	4.5	11.6	10.7	27.4
12	38.7	21.1	54.8	0.52	1.3	1.47	3.8	0.73	1.7	3.4	8.8	11.4	29.5
13	38.7	16.9	43.5	0.50	1.3	1.25	3.2	0.96	2.5	5.0	12.9	14.1	36.5
14	38.7	19.8	51.1	0.60	1.6	1.25	3.2	0.83	2.1	4.5	11.6	11.7	30.2
15	38.4	17.6	45.8	0.48	1.3	1.41	4.1	1.16	3.0	4.7	12.1	13.1	34.1
16	38.4	18.5	48.2	0.44	1.1	1.78	4.6	0.87	2.3	3.9	10.2	12.9	33.6
17	38.1	21.4	56.1	0.48	1.3	1.16	3.1	0.93	2.4	4.1	10.8	10.0	26.3
18	37.8	18.2	48.3	0.48	1.3	1.15	3.0	0.83	2.2	4.7	12.4	12.4	32.8
19	37.5	15.9	42.4	0.48	1.3	1.41	3.8	0.83	2.2	4.8	12.8	14.1	37.6
20	37.5	15.5	41.3	0.52	1.4	1.22	3.3	0.73	1.9	6.1	16.3	13.4	35.7
21	37.3	21.3	57.1	0.53	1.4	1.38	3.7	0.82	2.2	5.7	15.3	7.6	20.4
22	37.3	19.2	51.6	0.44	1.2	1.44	3.9	0.56	1.5	6.0	16.0	9.6	25.8
23	36.6	22.0	60.1	0.41	1.1	1.44	3.9	0.80	2.2	5.4	14.7	6.6	18.0
24	36.3	17.5	48.2	0.44	1.2	1.22	3.4	1.03	2.8	4.8	13.2	11.3	31.1
25	36.0	13.3	37.0	0.44	1.2	1.60	4.4	1.00	2.8	7.2	20.0	12.5	34.7
26	35.9	15.3	42.6	0.48	1.3	1.06	3.0	0.67	1.9	4.1	11.4	14.3	38.8
27	35.9	23.6	65.7	0.54	1.5	1.48	4.1	0.69	1.9	5.9	16.5	3.7	10.4
28	35.7	16.4	45.9	0.44	1.2	1.31	3.7	0.70	2.0	4.8	13.4	12.0	33.6
29	35.4	18.4	52.0	0.44	1.2	1.35	3.8	0.90	2.5	5.0	14.1	9.3	26.3
30	34.8	15.0	43.1	0.48	1.4	1.52	4.4	0.70	2.0	4.6	13.2	12.5	35.9
31	34.8	15.5	44.3	0.41	1.2	0.99	2.8	0.83	2.4	3.6	10.3	13.6	38.8
32	34.6	13.1	37.9	0.43	1.2	1.19	3.4	0.70	2.0	5.3	15.3	13.9	40.2
33	34.5	17.7	51.3	0.48	1.4	1.31	3.8	1.07	3.1	5.1	14.8	8.8	25.6
34	34.5	17.7	51.2	0.40	1.2	1.45	4.2	0.61	1.8	5.6	16.1	8.8	25.6
35	34.2	11.8	34.5	0.44	1.3	0.96	2.5	0.70	2.1	3.7	10.8	16.6	48.6
36	33.9	15.1	44.5	0.48	1.4	0.93	2.7	0.80	2.3	5.5	15.6	11.2	33.0
37	33.7	16.6	49.2	0.46	1.4	1.20	3.6	0.79	2.3	6.1	18.1	8.6	25.5
38	33.7	16.7	49.5	0.48	1.4	1.48	4.4	0.73	2.2	6.6	19.5	7.8	23.1

TABLE VI—*Concluded.*

No.	Non-protein N.	Urea N.		Creatinine N.		Creatine N.		Uric acid N.		Amino-acid N.		Rest N.	
	mg.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
39	33.6	17.7	52.7	0.44	1.3	0.96	2.9	0.73	2.3	5.7	17.0	8.1	24.3
40	33.6	11.1	33.0	0.41	1.2	1.38	4.1	0.63	1.9	4.0	11.9	16.1	48.1
41	33.6	13.3	39.6	0.52	1.5	1.35	4.1	1.16	3.5	5.6	16.7	11.6	34.5
42	33.1	15.9	47.9	0.48	1.4	1.42	4.3	0.79	2.4	3.6	10.9	11.0	33.1
43	33.0	12.2	37.0	0.52	1.6	1.38	4.2	0.67	2.0	4.0	12.1	14.2	43.0
44	33.0	15.4	46.7	0.48	1.5	1.19	3.6	0.70	2.1	5.4	16.4	9.8	29.8
45	33.0	12.3	37.3	0.52	1.6	1.35	4.2	0.77	2.3	4.0	12.1	14.1	42.7
46	32.8	18.3	55.8	0.48	1.5	1.35	4.1	0.70	2.1	3.6	11.0	8.4	25.6
47	32.4	16.6	51.3	0.48	1.5	1.60	4.9	0.67	2.1	5.3	16.4	7.7	23.8
48	32.4	14.8	46.0	0.41	1.3	1.47	4.5	0.50	1.5	4.3	13.3	10.8	33.3
49	32.4	15.1	46.6	0.48	1.5	1.41	4.4	0.53	1.6	4.8	14.8	10.1	31.5
50	32.4	16.3	50.3	0.52	1.6	1.12	3.5	0.70	2.2	3.9	12.0	9.9	30.6
51	32.1	16.6	51.7	0.44	1.4	1.16	3.6	0.87	2.7	4.7	14.6	8.3	25.9
52	31.8	12.8	40.3	0.44	1.4	1.38	4.3	0.73	2.5	5.0	15.7	11.4	35.8
53	31.7	17.0	53.7	0.43	1.4	1.03	3.3	0.60	1.9	3.9	12.4	8.7	27.4
54	31.5	17.6	55.9	0.44	1.4	1.15	3.6	0.70	2.2	5.0	15.9	6.6	20.9
55	30.6	12.5	40.9	0.48	1.6	1.35	4.4	1.10	3.6	6.7	21.9	8.5	27.8
56	30.0	14.4	48.0	0.48	1.6	1.16	3.9	0.83	2.8	4.1	13.7	9.0	30.0
57	29.7	13.3	44.1	0.41	1.4	1.25	4.2	0.90	3.0	3.1	10.4	10.9	36.7
58	28.8	11.3	39.2	0.48	1.7	1.28	4.4	0.73	2.5	5.0	17.4	10.0	34.7
59	28.2	15.8	55.9	0.48	1.7	0.63	2.2	0.65	2.3	3.9	13.9	6.7	23.8
60	27.3	9.7	35.5	0.37	1.4	1.35	4.9	0.57	2.1	4.3	15.8	11.0	40.3
Average...	35.6	17.1	47.8	0.47	1.3	1.30	3.7	0.78	2.2	4.9	13.8	11.0	31.1

If instead of the concentrations we consider the percentages of the total non-protein nitrogen which they represent, it is evident that in the blood as in the urine (33) the distribution of the nitrogen among the urea and the other nitrogenous constituents is dependent upon the absolute amounts of the total non-protein nitrogen present. For not only does the urea nitrogen tend to decrease in absolute amounts but also the percentage of the total non-protein nitrogen as urea nitrogen tends to decrease with the fall in the absolute amounts of the total non-protein nitrogen. This fall in percentage of urea nitrogen indicates that with falling total non-protein nitrogen the blood urea tends to decrease in concentration to a relatively greater extent than do the other constituents.

In order to bring out the existence of any specific relations between one constituent and the others, the absolute amounts of each component were arranged in the order of their descending value without regard to origin and compared with the corresponding values of the other constituents. It was found that when the urea nitrogen was used as the basis for comparison the only significant relation to be observed lay in the fact that there was a tendency for the rest nitrogen to increase in absolute amounts with the decrease in the urea nitrogen.

Similar comparisons using the creatinine, creatine, uric acid, and amino-acid nitrogen respectively as a basis have revealed no significant interrelations. The ratio between the creatinine and creatine nitrogen is quite variable, the creatinine nitrogen ranging from 24 to 76 per cent of the creatine nitrogen as the extreme limits, the common limits being from 27 to 46 per cent. About 13 per cent of the values of the ratio lie between 24 and 28 per cent, 77 per cent between 30 and 45 per cent, and 10 per cent between 46 and 76 per cent.

SUMMARY.

These studies of the chemical composition of human blood indicate:

1. While the total nitrogen, non-protein nitrogenous constituents, and the sugar of the blood vary in the same individual from week to week, there is a tendency for the level of these variations to be characteristically individual.

2. The sum of the average deviations of the constituents for any given individual may be an index of the metabolic stability of that individual.

3. The order of relative variability of the constituents determined is: creatinine, total non-protein nitrogen, total nitrogen, creatine, sugar, uric acid, amino-acid, urea, and rest nitrogen. A rough division can be made into groups of low, intermediate, and high variability.

4. There is no practical difference between the absolute amounts of the constituents found in bloods taken 14 hours after eating, *i.e.* before breaskfast, and 3½ hours after this meal. The slightly lower values found before breakfast for the non-protein, urea, and uric acid nitrogen are taken as meaning a lessened metabolism.

5. The absolute amounts of the urea, creatine, uric acid, amino-acid, and rest nitrogen tend to decrease with decrease in level of the total non-protein nitrogen. The urea decreases to relatively the greatest extent.

6. The absolute amount of creatinine is a constant for the individual and for the species. It is independent of quantitative changes in the level of the non-protein nitrogen; of the changes in the concentrations of any of the other constituents determined; and of the individual variations in metabolic stability.

7. There is no uniform quantitative relation between the amounts of the creatinine and creatine nitrogen in the blood.

8. The distribution of the non-protein nitrogen among urea and the other soluble nitrogenous constituents of the blood is dependent to a great degree upon the absolute amounts of the total non-protein nitrogen present.

9. The nitrogenous constituents of blood that are commonly found in the urine, *e.g.* urea, creatinine, and uric acid, seem to undergo the same type of absolute and relative change with change in level of the total non-protein nitrogen of the blood as they do with the change from the high to low total nitrogen in the urine.

10. The relative as well as the absolute decline in the urea nitrogen of the blood accompanying the decrease in the total non-protein nitrogen is compensated for by a relatively lesser absolute decrease in all the other nitrogenous constituents, which results in an increase of the percentage of their nitrogen in terms of the non-protein nitrogen.

11. The only apparent interrelation existing between any of the individual constituents is that between the urea and the rest nitrogen. There is a tendency for a rise or fall in the blood urea to be accompanied by a change in the opposite direction of the rest nitrogen.

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THE HEAT COAGULATION OF MILK.

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In our article with the above title published in this *Journal* in November, 1919, the temperature of 136°C. was chosen as a suitable one for the detection of milks liable to coagulate in the condensing process. Mr. George Grindrod, now with the Carnation Milk Products Company of Oconomowoc, Wisconsin, was the first to employ high temperatures in a "heat test" as suitable for the detection of such condition and to have used such temperatures with the autoclave. In our paper we failed to give Mr. Grindrod credit for having made use of this means of testing milk for coagulation effects. We now wish to correct this error of omission.

In our paper we mentioned the disadvantage of using the autoclave for heating the milk and reported on our modification of using sealed tubes in a zylene bath at 136°C. In our later correspondence with Mr. Grindrod he claims that he had also used the sealed tube method for a period of 3 years, although he had not published any description of this test or given us any information of a similar import.

A NEW 0.1 N CALOMEL ELECTRODE DESIGN.

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In accurate H ion concentration work the 0.1 N calomel electrode is nearly universally used, mainly because its E. M. F. is not nearly so readily affected by temperature change as the saturated type.

Furthermore, a saturated KCl solution, unless in a tightly sealed vessel, is disagreeable to work with because of the tendency of the salt to creep from the solution.

However, it is apparent that the 0.1 N electrode can be used to advantage only when the normality remains perfectly constant. When the 0.1 N electrode is used with the necessary saturated KCl bridge, this becomes a problem.

Various appliances are in use and practically all depend on a very small opening at the end of the side-arm of the calomel electrode. Diffusion is only hindered at best and the arm has to be washed out with the solution from the electrode vessel. Furthermore, the side-arm must remain in the saturated KCl solution only when necessary, so the arrangement is not permanent.

We are using a design in several H ion concentration apparatus which seems to overcome these difficulties completely. Its use was practically imperative in a problem where several determinations were made each day over a long period of time, inasmuch as the electrode chain could be set up permanently without any change in normality of the 0.1 N KCl or any creeping of salts.

During a period of nearly 2 months of continual use the E. M. F. of the calomel electrode had not changed in the least.

The design can readily be understood from the diagram. The apparatus is clamped to a horizontal bar and the lower tube from stop-cock C connected by a large flexible rubber tube to

the Clark or McClendon electrode mounted on the usual shaking arm. The vertical arm from stop-cock C is connected to a bottle of saturated KCl solution which usually is placed well back of the apparatus so as to be out of the way. The same is true of the bottle of 0.1 N KCl solution which is connected to the other vertical arm.

Contact is made between the 0.1 N KCl and the saturated KCl solution around the closed, ungreased stop-cock B. Stop-cock A, which is greased with pure vaseline, is opened so as to connect the

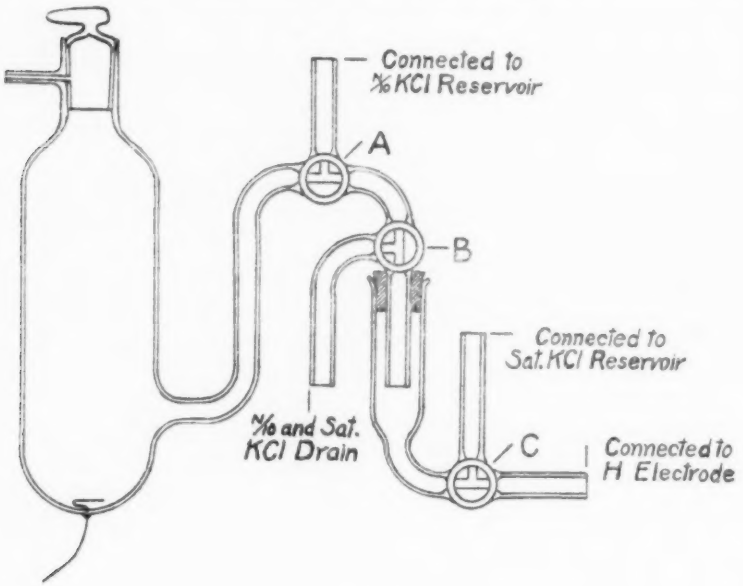

FIG. 1

electrode vessel with the bridge *only* when making a reading. Immediately after making a reading by the proper manipulation of stop-cocks A and B the section from A to B is thoroughly washed out through the side drain arm with fresh 0.1 N KCl from the reservoir.

In a similar manner any 0.1 N KCl can be washed from the saturated KCl below stop-cock B.

This apparatus thus gives a permanently closed system which minimizes contamination of the 0.1 N solution of the calomel electrode.

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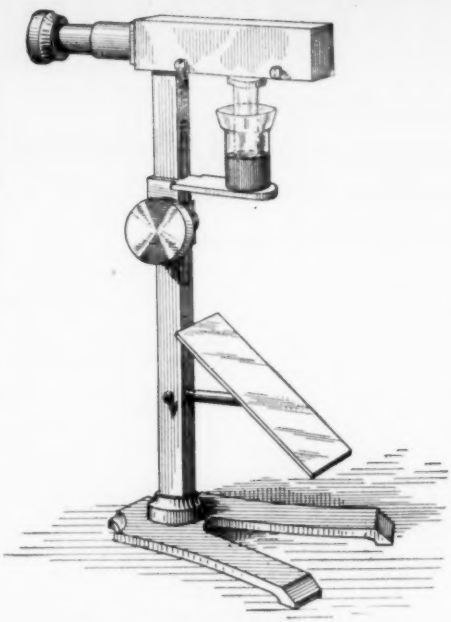

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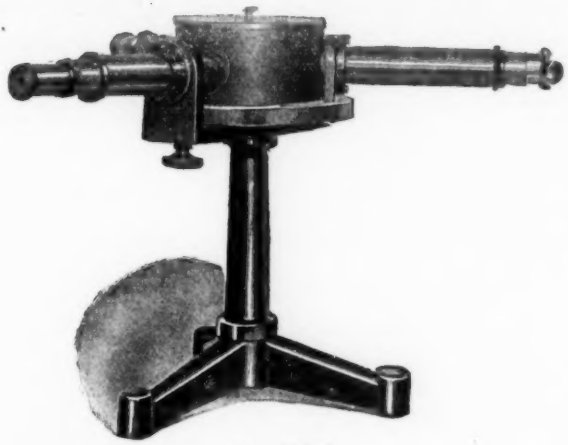

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